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#18

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Michael Wayne Graham et al.	Examiner: Sumesh Kaushal
Serial No.: 09/100,812	Art Unit: 1633
Filed: 19 June 1998	
For: Synthetic genes and genetic constructs comprising same I	DECLARATION OF: Michael Wayne Graham

Commissioner of Patents and Trademarks
Washington D.C. 20231

Sir,

I, Michael Wayne Graham, state as follows:

My present position is Principal Research Scientist of Benitec Australia Ltd, one of the assignees of the subject application. I am authorised to make this declaration on behalf of the applicants. I hold a Ph.D. in molecular biology and previously worked as a scientific investigator for several other scientific organisations. Now produced and shown to me marked MWG1 is my current resume and publications list.

I have read the above-captioned application and followed the prosecution thereof. I understand the Examiner has contended that the specification does not provide sufficient working examples that demonstrate that the expression of a synthetic gene in a cell results in the delay, repression or reduction of the expression of a target gene.

In my opinion, a scientist of routine skill, who was familiar with standard methods of molecular biology and biochemistry, would have been able to repress, delay or otherwise reduce expression of a target gene in an animal cell from the teaching of the specification.

My colleagues and I have carried out the following experiments which resulted in the expression of a synthetic gene or genetic construct in an animal cell causing the delay, repression or reduction (also called "silencing") of expression of a target gene. The methods used in these experiments to generate the data set out below are either in substance those taught in the specification or readily available to a skilled addressee in

the art as at 19 June 1998. I refer to the original specification as filed in June 1998 of to this application as the "Specification".

Co-suppression of Bovine Enterovirus (BEV) in Madin Darby Bovine Kidney Type CRIB-1 cells in vitro

CRIB-1 cells (derived from bovine kidney epithelial cells) were grown as adherent monolayers using DMEM supplemented with 10% v/v Donor Calf Serum (DCS; Life Technologies). The plasmid pCR.BEV2 was constructed as per page 29, lines 6 to 12 of the specification, and used to generate the two genetic constructs used in this experiment, pCMV.BEV2.GFP.2VEB and pCMV.BEV2.BGI2.2VEB. Both were constructed with an inverted repeat or palindrome of the BEV2 polymerase coding region that is interrupted by the insertion of either the GFP sequence (see page 32 paragraph 9 of the specification) or the sequence of the human β globin intron 2, as a stuffer fragment, using methods known in the art and as taught in the original specification. The human β-globin intron 2 sequences were isolated using PCR amplification of human genomic DNA to generate the stuffer fragment using methods known in the art and as taught in the original specification.

To detect a co-suppression phenotype, CRIB-1 cells were transformed with the Bovine enterovirus RNA genetic constructs pCMV.BEV2.GFP.2VEB and pCMV.BEV2.BGI2.2VEB. Transformations were performed in 6-well tissue culture vessels. Individual wells were seeded with 2 x 10^5 CRIB-1 cells in 2 ml of DMEM, 10% v/v DCS and incubated at 37°C in 5% v/v CO_2 until the monolayer was 60–90% confluent, typically 16–24 hr. The following solutions were prepared in 10 ml sterile tubes:

Solution A: For each transfection, 1 µg of DNA (pCMV.BEV2.GFP.2VEB or pCMV.BEV2.BGI2.2VEB) was diluted into 100 µl of Opti-MEM-I (registered trademark) and;

Solution B: For each transfection, 10 µl of LipofectAMINE (trademark) Reagent (Life Technologies) was diluted into 100 µl Opti-MEM-I (registered trademark).

The two solutions were combined and mixed gently, and incubated at room temperature for 45 min to allow DNA-liposome complexes to form. While complexes formed, the CRIB-1 cells were rinsed once with 2 ml of Opti-MEM I (registered trademark). For each transfection, 0.8 ml of Opti-MEM I (registered trademark) was added to the tube containing the complexes, the tube mixed gently, and the diluted complex solution overlaid onto the rinsed CRIB-1 cells. Cells were then incubated with the complexes at 37°C in 5% v/v CO_2 for 16–24 hr. Transfection mixture was then removed and the CRIB-1 monolayers overlaid with 2 ml of DMEM, 10% v/v DCS. Cells were incubated at 37°C in 5% v/v CO_2 for approximately 48 hr. To select for stable transformants, the medium was replaced every 72 hr with 4 ml of DMEM, 10% v/v DCS, 0.6 mg/ml geneticin. After 21 days of selection, stably transformed CRIB-1 colonies were apparent. Individual colonies of stably transfected CRIB-1 cells were cloned, maintained and stored as described in the previous example.

The bovine enterovirus stock used in these experiments were derived from a cloned isolate, K2577. To amplify BEV virus from this stock, cells were infected in 6-well culture vessels with 5 µl of viral stock per well and the virus allowed to replicate for 48 hrs. Culture medium was harvested at this time and transferred to a screw-capped tube. Dead cells and debris were removed by centrifugation at 3,500 rpm for 15 min at 4°C in a Sigma 3K18 centrifuge. The supernatant was decanted into a fresh tube and centrifuged at 20,000 rpm for 30 min at 4°C in a Beckman J2-M1 centrifuge to remove remaining debris. The supernatant was decanted and this new BEV stock titred as described below and stored at 4°C.

To titre virus, a 24-well tissue culture plate was seeded with 4 x 10^4 CRIB-1 cells in 800 µl DMEM, 10% v/v DCS. The cells were incubated at 37°C in 5% v/v CO_2 until 90–100% confluent. From concentrated BEV viral stock, virus was diluted in serum-free DMEM at dilutions of 10^{-1} to 10^{-9}. The medium was aspirated from the CRIB-1 monolayers and the monolayers overlaid with 800 µl of 1 x PBS and washed by gently rocking the tissue culture vessel. PBS was aspirated from the monolayers and the wash repeated. 200 µl of the diluted virus solutions (10^{-3} to 10^{-9}) was added immediately directly onto the rinsed CRIB-1 cells using one dilution per well in duplicate. The CRIB-1 cells were incubated with BEV for 24 hr at 37°C in 5% v/v

CO_2 and each well inspected microscopically for cell lysis. A further 600 µl of serum-free DMEM was then added to each well. After a further 24 hr, each well was inspected microscopically for cell lysis. The working dilution is the minimum viral concentration that kills most of the CRIB-1 cells after 24 hr and all cells after 48 hr.

The CRIB-1 cells transformed with pCMV.BEV2.GFP.2VEB or pCMV.BEV2.BGI2.2VEB were challenged with bovine enterovirus as follows. In a 24-well tissue culture plate, 4×10^4 CRIB-1 cells per well were seeded in triplicate, in 800 µl DMEM, 10% v/v DCS. The cells were incubated at 37°C in 5% v/v CO_2 until 90–100% confluent. From concentrated BEV viral stock, BEV virus was diluted in serum-free DMEM at an appropriate dilution. In addition, the BEV viral stock was tested at 10x and 0.1x the working dilution (typically 10^{-4} to 10^{-6} fold dilutions). Medium was aspirated from the CRIB-1 monolayers and the cells overlaid with 800 µl of 1 x PBS and washed gently by rocking the tissue culture vessel. PBS was aspirated from the monolayers and the wash repeated. 200 µl of the diluted virus solutions (one dilution per replicate) was added immediately, directly onto the rinsed CRIB-1 cells. The cells were incubated with BEV for 24 hr at 37°C in 5% v/v CO_2, and each well inspected microscopically for cell lysis. A further 600 µl of serum-free DMEM was added to each well. After a further 24 hr, each well was inspected microscopically for cell lysis.

To determine whether cells transformed with pCMV.BEV2.GFP.2VEB or pCMV.BEV2.BGI2.2VEB became tolerant to BEV infection (ie demonstrate silencing of the BEV polymerase structural gene), transformed cell lines were challenged with dilutions of viral stock and cell survival monitored. To overcome inherent variation in these assays, multiple challenges were performed and lines consistently showing viral tolerance were isolated for further examination. Results of these experiments are shown below in the two tables in Annexure MWG2, one table each for lines transformed with the genetic constructs containing the GFP or BGI2 stuffer fragments respectively. These data showed that viral-tolerant cell lines could be generated in this fashion using either construct, although the degree of silencing varied between cell lines and between replicate viral challenges. Some cell lines

showed consistent survival in repeated challenges, ie strong silencing. In addition, cells which survived this viral challenge could be grown up for further analyses.

To further define the degree of viral tolerance in such cell lines, the cell line CRIB-1 BGI2 #19, and viral-tolerant cells grown from cells that survived the initial challenge (line CRIB-1 BGI2 #19(tol)), were further analysed using finer scale (3-fold) serial dilutions of BEV in triplicate. The results of these experiments are shown in Annexure MWG3. These data showed that the cell lines CRIB-1 BGI2 #19 and CRIB-1 BGI2 #19(tol) were tolerant to higher titres of BEV than the parental CRIB-1 line. Annexure MWG4 shows micrographs of CRIB-1 cells and a CRIB-1 transformed line [CRIB-1 BGI2 # 19(tol)] prior to and 48 hrs after infection with identical titres of BEV.

(a) is CRIB-1 cells prior to BEV infection;

(b) is CRIB-1 cells 48 hrs after BEV infection;

(c) is CRIB-1 BGI2 # 19(tol) cells prior to infection with BEV;

(d) is CRIB-1 BGI2 # 19(tol) 48 hrs after BEV infection.

It is therefore apparent that each of the plasmids pCMV.BEV2.GFP.2VEB and pCMV.BEV2.BGI2.2VEB is a genetic construct (which has a nucleotide sequence, BEV2, substantially identical to a target gene, BEV polymerase, in an animal cell, operably linked with a promoter) that represses expression of that target gene, the BEV polymerase of the viral genome, when it is expressed in the animal cell.

Co-suppression of Tyrosinase in Murine Type B16 cells in vitro

B16 cells were derived from murine melanoma (ATCC CRL-6322) and grown as adherent monolayers in RPMI 1640 supplemented with 10% v/v FBS. Since these cells are derived from melanocytes, they normally express tyrosinase and contain melanin pigment. To demonstrate tyrosinase silencing, genetic constructs were made analogous to the constructs of Figures 13 and 15 of the Specification, but of course using a part of the TYR gene, in a similar manner to Figure 23 of the Specification.

First, plasmid TOPO.TYR was generated, by purifying total RNA from cultured murine B16 melanoma cells, cDNA was prepared from this and a region of the murine tyrosinase gene amplified by PCR using the primers:

TYR-F: GTT TCC AGA TCT CTG ATG GC

and

TYR-R: AGT CCA CTC TGG ATC CTA GG.

The resultant fragment was cloned into pCR (registered trademark) 2.1-TOPO according to the supplier's instructions (Invitrogen) to make plasmid TOPO.TYR. Plasmid pCMV.TYR.BGI2.RYT was constructed with an inverted repeat, or palindrome, of a region of the murine tyrosinase gene that is interrupted by the insertion of the same human β-globin intron 2 (BGI2) sequence described above. Plasmid pCMV.TYR.BGI2.RYT was constructed in successive steps: (i) the human BGI2 sequences were cloned into the polylinker of pCMV.cass (see Figure 2 of the Specification) to generate pCMV.BGI2 (ii) the TYR sequence from plasmid TOPO.TYR was sub-cloned in the sense orientation as a BglII-to-BamHI fragment into BglII-digested pCMV.BGI2 to make plasmid pCMV.TYR.BGI2, and (iii) the TYR sequence from plasmid TOPO.TYR was sub-cloned in the antisense orientation as a BglII-to-BamHI fragment into BamHI-digested pCMV.TYR.BGI2 to make plasmid pCMV.TYR.BGI2.RYT.

Plasmid pCMV.TYR.TYR was constructed with a direct repeat of the mouse tyrosinase cDNA sequence, expression of which is driven by the CMV promoter. Plasmid pCMV.TYR.TYR was constructed by cloning the TYR sequence from plasmid TOPO.TYR as a BamHI-to-BglII fragment into BamHI-digested pCMV.TYR (itself constructed by cloning the TYR sequence from plasmid TOPO.TYR as a BamHI-to-BglII fragment into BamHI-digested pCMV.cass and selecting plasmids containing the TYR sequence in a sense orientation relative to the CMV promoter) and selecting plasmids containing the second TYR sequence in a sense orientation relative to the CMV promoter.

The tyrosinase silencing phenotype was detected by assaying for reduction of melanin pigmentation following insertion of the genetic constructs pCMV.TYR.BGI2.RYT or pCMV.TYR.TYR into murine melanoma B16 cells. Tyrosinase is the major enzyme controlling pigmentation in mammals. If the gene is inactivated, melanin will no longer be produced by the pigmented B16 melanoma cells. This is essentially the same process that occurs in albino animals.

Transformations were performed in 6-well tissue culture vessels. Individual wells were seeded with 1 x 10^5 cells in 2 ml of RPMI 1640, 10% v/v FBS and incubated at 37°C in 5% v/v CO_2 until the monolayer was 60–90% confluent, typically 16–24 hr. Subsequent procedures were as described above, except that B16 cells were incubated with the DNA-liposome complexes at 37°C in 5% v/v CO_2 for 3–4 hr only. Individual colonies of stably transfected B16 cells were cloned and 36 clones stably transformed with pCMV.TYR.BGI2.RYT and 37 clones stably transformed with pCMV.TYR.TYR were selected for subsequent analyses. When expression of the endogenous tyrosinase gene is reduced, melanin production in the B16 cells is also reduced. B16 cells that would normally appear to contain a dark brown pigment will now appear lightly pigmented or unpigmented.

To monitor visually melanin content of transformed B16 cell lines, cells were trypsinized and transferred to media containing FBS to inhibit trypsin activity. Cells were then counted with a haemocytometer and 2 x 10^6 cells transferred to a microfuge tube. Cells were collected by centrifugation at 2,500 rpm for 3 mins at room temperature and pellets examined visually. As can be seen from the results photographed in Annexure MWG5, five of the cloned cell lines transformed with pCMV.TYR.BGI2.RYT, namely B16 2 1.11, B16 3.1.4, B16 3.1.15, B16 4.12.2 and B16 4.12.3, were considerably paler than the B16 controls. Five clones transformed with pCMV.TYR.TYR (B16+TyrTyr 1.1, B16+TyrTyr 2.9, B16+TyrTyr 3.7, B16+TyrTyr 3.13 and B16+TyrTyr 4.4) were also significantly paler than the B16 controls.

Another assay for measuring target gene (tyrosinase) repression was as follows. Specific diagnosis for the presence of cellular melanin can be achieved using a modified Schmorl's melanin staining (Koss 1979 Diagnostic Cytology. Ed. J.P.

Lippincott). Using this method, the presence of melanin in the cell is detected by a specific staining procedure that converts melanin to a greenish-black pigment. The results of staining with Schmorl's stain correlated with the simple visual data illustrated in Annexure MWG5 for all cell lines. When B16 cells were stained with the above procedure, melanin was obvious in most cells. In contrast, fewer cells stained for melanin in the transformed lines B16 2.1.11, B16 3.1.4, B16 3.1.15, B16 4.12.2, B16 4.12.3, B16 TyrTyr 1.1, B16 TyrTyr 2.9 and B16 TyrTyr 3.7, consistent with the reduced gross pigmentation observed in these cell lines.

Tyrosinase repression in transformed cell lines was also assessed utilising tyrosinase enzyme assays where the DOPA oxidase activity of cell extracts was determined. This assay uses Besthorn's hydrazone (3-methyl-2-benzothiazolinonehydrazone hydrochloride, MBTH) to trap dopaquinone formed by the oxidation of L-dopa and rate of production of the pink pigment can be used as a quantitative measure of enzyme levels (Winder and Harris 1991, European Journal of Biochemistry, 198: 317-326; Dutkiewicz, et al., 2000, Experimental Eye Research, 70: 563-569).

B16 cells and transformed B16 cell lines were plated into individual wells of a 96-well plate in triplicate. Constant numbers of cells (25,000) were transferred into individual wells and were incubated overnight in RPMI 1640 supplemented with 10% v/v FBS at 37°C in 5% v/v CO_2. Tyrosinase assays were performed (as described below) after either 24 or 48 hr incubation. Individual wells were washed with 200 µl PBS and 20 µl of 0.5% v/v Triton X-100 in 50mM sodium phosphate buffer (pH 6.9) was added to each well. Cell lysis and solubilisation was achieved by freeze-thawing plates at -70°C for 30 min, followed by incubating at room temperature for 25 min and 37°C for 5 min.

Tyrosinase activity was assayed by adding 190 µl freshly-prepared assay buffer (6.3mM MBTH, 1.1mM L-dopa, 4% v/v N,N'-dimethylformamide in 48mM sodium phosphate buffer (pH 7.1)) to each well. Colour formation was monitored at 505 nm in a Tecan plate reader and data collected using X/Scan Software. Readings were taken at constant time intervals and reactions monitored at room temperature, typically 22°C. Data were analysed and tyrosinase activity estimated at early time-points when product formation was linear, typically between 2 and 12 min. Results

were calculated as the average of enzyme activities as measured for the triplicate samples. Results from these experiments are shown below in Annexure MWG6. These data showed that tyrosinase enzyme activity was reduced in lines transformed with the constructs pCMV.TYR.BGI2.RYT and pCMV.TYR.TYR.

These assays therefore show that each of the plasmids pCMV.TYR.GFP.RYT and pCMV.TYR.TYR is a genetic construct (which has a nucleotide sequence identical to a structural target gene, tyrosinase, of an animal cell, operably linked with a promoter) represses expression of that target gene, the endogenous mouse tyrosinase gene, when it is expressed in an animal cell.

Additional Examples

We then performed similar experiments to the two described above on a range of target genes using the target genes, cell lines and assays set out in the Table below. In these experiments, genetic constructs were generated in the same manner as that described above using techniques known to one skilled in the art, using the CMV promoter in a plasmid, with the same BGI2 stuffer fragment between the two palindromic forms of a portion of the synthetic gene which is substantially identical to the target gene. Plasmid maps of these constructs are annexed as set out in the table below. The constructs were then introduced into the animal cell lines as identified in the table below and transcribed by the host cell's natural mechanisms. In each case, repression of the target structural gene was found, using assays described by others and known by those skilled in the art.

Gene	Cell Line	Assay	Plasmid Map Annexure
Galactosyl Transferase (GalT)	PK-1	IB-4 binding	MWG7
Her-2	MDA-MB-468	Antibody binding	MWG8
YB-1	B10.2 and Pam 212	Cell death	MWG9
p53	B10.2 and Pam 212	Cell death	MWG10
CD4	Jurkatt	Antibody binding	MWG11

A brief summary of each assay protocol is set out below.

GalT

Galactosyl transferase (GalT) catalyses the addition of galactosyl residues to cell surface proteins. GalT activity can be most conveniently assayed using a plant lectin (IB4), which binds specifically to galactosyl residues on cell surface proteins. To detect IB4 binding, fluorescence assays were used. Cells were fixed, then probed with IB4 conjugated with biotin (Sigma) followed by streptavidin conjugated FITC. GalT inactivation was monitored using either fluorescence microscopy or FACS.

HER-2

HER-2 (also designated neu and erbB-2) encodes a 185 kDa transmembrane receptor tyrosine kinase that is constitutively activated at low levels and displays potent oncogenic activity when over-expressed. The level of HER-2 protein in cells can be monitored using antibody probes, fluorescence can be monitored by flow cytometry or fluorescence microscopy.

To determine the level of expression of HER-2 in transformed cell lines, using flow cytometry, approximately 500,000 cells grown in a 6-well plate were washed twice with 1 x PBS then dissociated with 500 µl cell dissociation solution (Sigma C 5789) according to the manufacturer's instructions (Sigma). Cells were transferred to medium in a microcentrifuge tube and collected by centifugation at 2,500 rpm for 3 min. The supernatant was removed and cells resuspended in 1 ml 1 x PBS.

For fixation, cells were collected by centrifugation as above and suspended in 50 µl PBA (1 x PBS, 0.1 % w/v BSA fraction V (Trace) and 0.1 % w/v sodium azide) followed by the addition of 250 µl of 4 % w/v paraformaldehyde in 1 x PBS, and incubated at 4°C for 10 min. To permeabilize cells, cells were collected by centrifugation at 10,000 rpm for 30 sec, the supernatant removed and cells suspended in 50 µl 0.25 % w/v saponin (Sigma S 4521) in PBA and incubated at 4°C for 10 min.

To block cells, cells were collected by centrifugation at 10,000 rpm for 30 sec, the supernatant removed and cells suspended in 50 µl PBA, 1 % v/v FBS and incubated at 4°C for 10 min.

To quantify HER-2 protein, fixed, permeabilized cells were probed with Anti-erbB2 monoclonal antibody (Transduction Laboratories) at 1/100 dilution followed by Alexa Fluor 488 goat anti-mouse IgG conjugate (Molecular Probes) at 1/100 dilution. Cells were then analysed by flow cytometry using a Becton Dickinson FACSCalibur and Cellquest software (Becton Dickinson). To estimate true background fluorescence values, unstained MDA-MB-468 cells, and cells probed with an irrelevant primary antibody (MART-1, an IgG2b antibody (NeoMarkers)) and the Alexa Fluor 488 secondary antibody, both at 1/100 dilutions.

Similar protocols for fixation and staining were used to monitor HER-2 expression in cell colonies.

Cell Death

YB-1 (Y-box DNA/RNA-binding factor 1) is a transcription factor that binds to the promoter region of the p53 gene and in so doing represses its expression. In cancer cells that express normal p53 protein at normal levels (some 50% of all human cancers), the expression of p53 is under the control of YB-1, such that diminution of YB-1 expression results in increased levels of p53 protein and consequent apoptosis. The murine cell lines B10.2 and Pam 212 are two such tumorigenic cell lines with normal p53 expression. The expected phenotype for co-suppression of YB-1 in these two cell lines is apoptosis.

To monitor apoptosis, live and dead cell numbers were determined by trypan blue staining (0.2%) and counting in quadruplicate on a haemocytometer slide.

CD4

CD4 is a cell surface antigen present on the surface of some classes T cells. Its levels were monitored by flow cytometry , using protocols similar to those described for HER-2.

In addition, other researchers have also shown in work published in 2000 and 2001, that similar constructs (ie plasmid constructs containing a promoter which drives expression of structural gene sequence palindromes) repress target gene expression *in vitro* in animal cells (Yang *et al*, 2001, . Molecular and Cellular Biology, 21: 7807-7816) and *in vivo* in transformed *Drosophila* (Tavernakis et al, 2000, Nature Genetics, 24: 180-183).

In addition, we generated a similar construct with the GalT synthetic gene but which comprised a direct tandem repeat of the GalT synthetic gene rather than a palindrome in the same manner to the pCMV.TYR.TYR construct described above using techniques known to one skilled in the art. This construct also repressed expression of the structural GalT gene in the same cell line. These experiments all consistently demonstrated to us the wide application of the repression techniques described in the specification.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge and wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of the application or any patent issuing thereon.

Date 23 April 2002 By _____
Michael Wayne Graham

Attached: Annexures MWG1 to MWG11 inclusive.

ANNEXURE MWG1

E.1.

CURRICULUM VITAE

PERSONAL DETAILS

NAME:	GRAHAM, Michael Wayne
DATE OF BIRTH:	3 January, 1958
PLACE OF BIRTH:	Sydney, Australia
MARITAL STATUS:	Married, three children

UNIVERSITY TRAINING

1981	B. Sc. (Hons.) Australian National University
1986	Ph. D. The University of Melbourne

POSITIONS HELD

1981-1982	Research Assistant, Department of Biochemistry, Australian National University
1982-1986	Postgraduate Student, Molecular Biology Unit, The Walter and Eliza Hall Institute of Medical Research, Melbourne
1986-1987	Research Officer, Agriculture and Forestry, University of Melbourne
1987-1991	Research Scientist, Calgene Pacific Pty. Ltd., Melbourne
1991-1997	Research Scientist, CSIRO Division of Plant Industry, Canberra
1997-2000	Principal Research Scientist, QDPI, QABC, Brisbane
2000-2001	Principal Research Scientist, Benitec Australia Ltd, Brisbane

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ANNEXURE MWG2

CRIB-1 cells transfected with pCMV.BEV.GFP.VEB (CRIB-1 GFP)

– no cells surviving
+ 1–10% of cells surviving.
++ 10–90% of cells surviving.
+++ 90%+ of cells surviving
nd not done.

Cell line	Challenge 1		Challenge 2		Challenge 3		Challenge 4	
	10^6	10^5	10^4	10^3	10^2	10^1	10^0	10^{-1}
CRIB-1	nd	nd	–	–	–	–	–	–
CRIB-1 GFP # 1	–	–	–	–	–	–	+	–
CRIB-1 GFP # 3	–	–	+	++	–	–	nd	nd
CRIB-1 GFP # 4	–	–	–	–	–	–	++	–
CRIB-1 GFP # 5	–	–	+	+++	–	–	nd	nd
CRIB-1 GFP # 6	–	+	–	–	–	–	–	–
CRIB-1 GFP # 7	+	+	–	+	+	+	nd	nd
CRIB-1 GFP # 8	+	+++	+	+	+	+++	–	++
CRIB-1 GFP # 9	–	–	–	+	+	+	nd	nd
CRIB-1 GFP # 10	–	+	–	+	+	++	nd	nd
CRIB-1 GFP # 11	+	++	–	–	+	+++	nd	nd
CRIB-1 GFP # 12	–	+	+	++	+	+	nd	nd
CRIB-1 GFP # 13	–	–	+	+	–	–	nd	nd
CRIB-1 GFP # 14	++	++	+	++	++	+	+	+
CRIB-1 GFP # 15	–	+	++	++	+	++	nd	nd
CRIB-1 GFP # 16	–	+	–	++	+	++	nd	nd
CRIB-1 GFP # 17	–	–	+	+	–	–	nd	nd
CRIB-1 GFP # 18	+	+	++	+	++	++	nd	nd
CRIB-1 GFP # 20	–	–	–	–	+	+++	nd	nd
CRIB-1 GFP # 21	–	++	+	++	+	+	nd	nd
CRIB-1 GFP # 22	–	+	+	+	+	+	nd	nd
CRIB-1 GFP # 23	–	–	–	+++	–	++	–	–
CRIB-1 GFP # 24	–	–	+	++	–	+		
CRIB-1 GFP # 25	–	+	–	+++	–	–	nd	nd
CRIB-1 GFP # 26	+	++	++	+++	++	+++	–	–

ANNEXURE MWG2

CRIB-1 cells transfected with pCMV.BEV2.BGI2.2VEB (CRIB-1 BGI2)

– no cells surviving
+ 1–10% of cells surviving.
++ 10–90% of cells surviving.
+++ 90%+ of cells surviving
nd not done.

Cell line	Challenge 1		Challenge 2		Challenge 3		Challenge 4	
CRIB-1	nd	nd	–	–	–	–	–	–
CRIB-1 BGI2 # 1	–	–	–	–	–	–	nd	nd
CRIB-1 BGI2 # 2	–	–	–	+	–	–	–	–
CRIB-1 BGI2 # 3	–	–	++	++	+	++	nd	nd
CRIB-1 BGI2 # 4	–	–	–	+	–	–	nd	nd
CRIB-1 BGI2 # 5	–	–	–	++	–	–	nd	nd
CRIB-1 BGI2 # 6	+	+	+++	++	+	+	nd	nd
CRIB-1 BGI2 # 7	+	+	–	+++	–	–	nd	nd
CRIB-1 BGI2 # 8	–	+	+++	++	–	+	nd	nd
CRIB-1 BGI2 # 9	–	+	–	++	+	++	–	++
CRIB-1 BGI2 # 10	++	++	++	+++	+	+	–	–
CRIB-1 BGI2 # 11	+	++	+	+	–	+	nd	nd
CRIB-1 BGI2 # 12	+	+	+	+++	–	–	nd	nd
CRIB-1 BGI2 # 13	–	–	+++	+++	–	–	nd	nd
CRIB-1 BGI2 # 14	+	++	+	++	+	+	nd	nd
CRIB-1 BGI2 # 15	+	+	+	++	+	++	–	–
CRIB-1 BGI2 # 16	–	–	–	–	–	–	nd	nd
CRIB-1 BGI2 # 17	–	+	–	++	–	–	nd	nd
CRIB-1 BGI2 # 18	–	–	–	+++	–	–	nd	nd
CRIB-1 BGI2 # 19	–	–	–	++	+	+++	+	+++
CRIB-1 BGI2 # 20	+	+	+	+++	+	+	nd	nd
CRIB-1 BGI2 # 21	–	–	–	–	–	–	–	–
CRIB-1 BGI2 # 22	–	–	–	–	–	–	–	–
CRIB-1 BGI2 # 23	–	+	+++	+++	+	+	nd	nd
CRIB-1 BGI2 # 24	–	++	+++	+	–	–	nd	nd

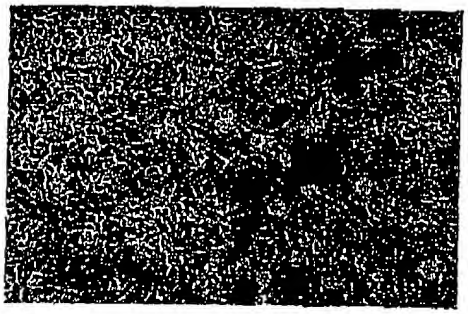
ANNEXURE MWG3

19

Cell line	Virus titre (pfu/ml)					
	1×10^3	1×10^4	5×10^4	1×10^5	5×10^5	1×10^6
CRIB-1 Replicate 1	–	–	–	–	–	+++
CRIB-1 Replicate 1	–	–	–	–	–	+
CRIB-1 Replicate 1	–	–	–	–	–	+++
CRIB-1 BGI2 #19 Replicate 1	–	–	+	+	++	+++
CRIB-1 BGI2 #19 Replicate 2	–	–	–	–	++	+++
CRIB-1 BGI2 #19 Replicate 3	–	–	–	+	+++	+++
CRIB-1 BGI2 #19(tol) Replicate 1	–	–	+	+	+++	+++
CRIB-1 BGI2 #19(tol) Replicate 2	–	–	+	+	++	+++
CRIB-1 BGI2 #19(tol) Replicate 3	–	–	+	+	+++	+++

ANNEXURE MWG4

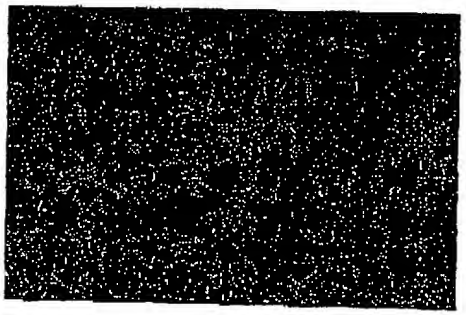
(a) CRIB-1 [0hrs]



(b) CRIB-1 [48hrs]

(c) CRIB-1 BGI2 #19(tol) [0hrs]

(d) CRIB-1 BGI2 #19(tol) [48hrs]

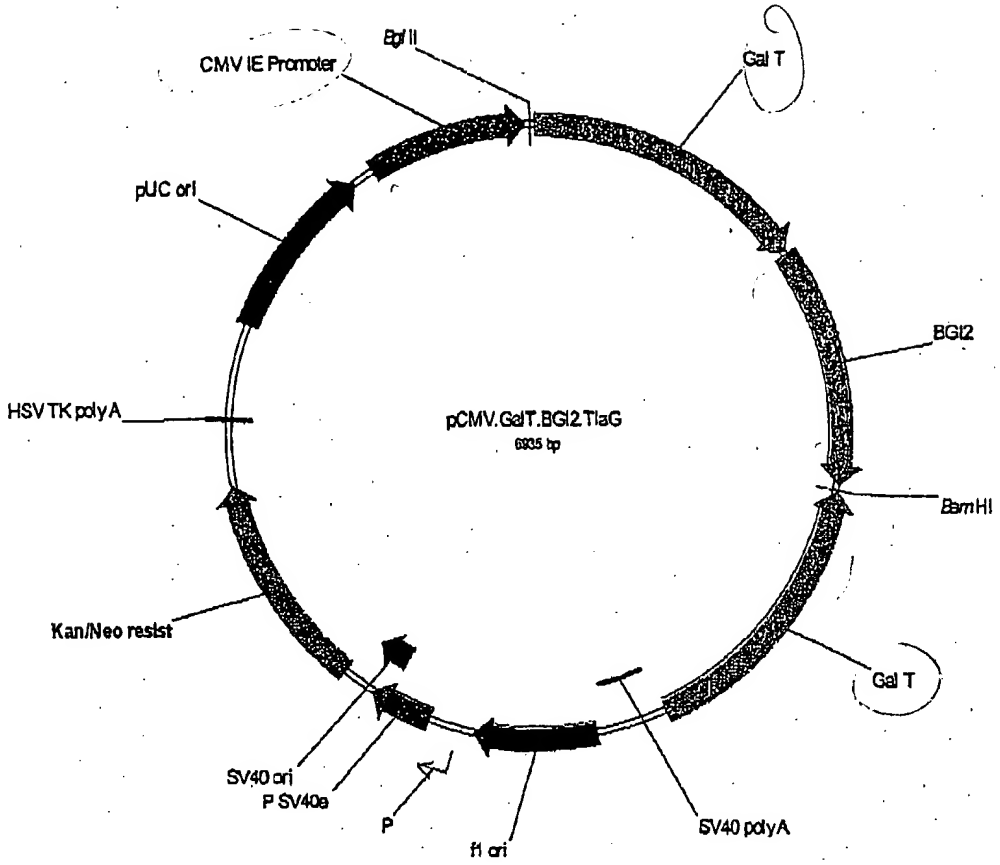


ANNEXURE MWG5

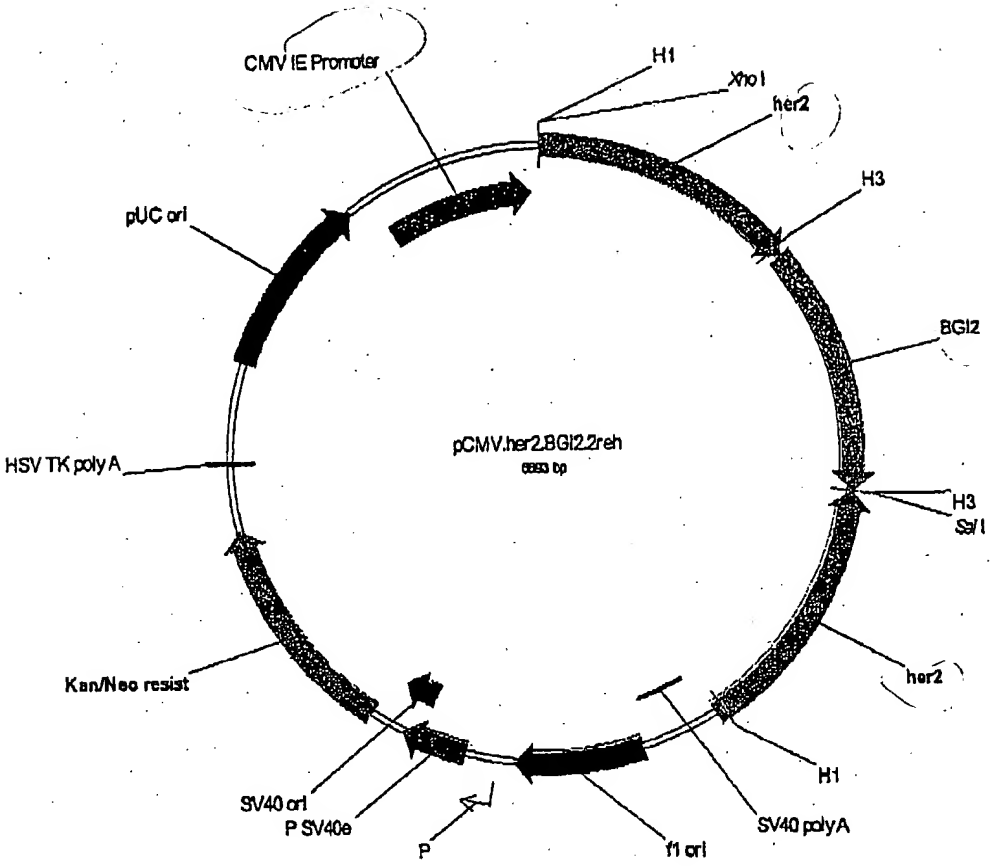
ANNEXURE MWG6

Cell Line	Tyrosinase activity ([illegible] 505 nm/[illegible] 25000 cells)	Relative tyrosinase activity compared with B16 cells (%)
B16	0.0123	100
B16 2.1.6 (Tyr.BGI2.ryT)	0.0108	87.8
B16 2.1.11 (Tyr.BGI2.ryT)	0.0007	5.7
B16 3.1.4 (Tyr.BGI2.ryT)	0.0033	26.8
B16 3.1.15 (Tyr.BGI2.ryT)	0.0011	8.9
B16 4.12.2 (Tyr.BGI2.ryT)	0.0013	10.6
B16 4.12.3 (Tyr.BGI2.ryT)	0.0011	8.9
B16 Tyr Tyr 1.1	0.0043	34
B16 Tyr Tyr 2.9	0.0042	34.1
B16 Tyr Tyr 3.7	0.0087	70.7

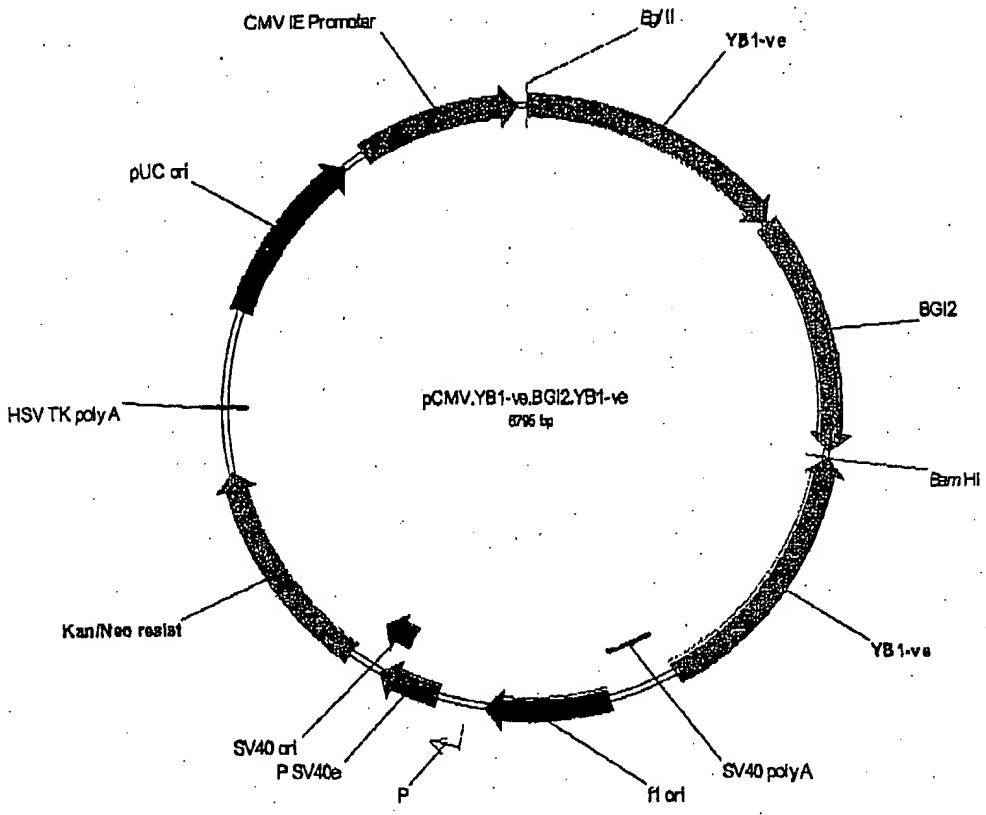
ANNEXURE MWG7



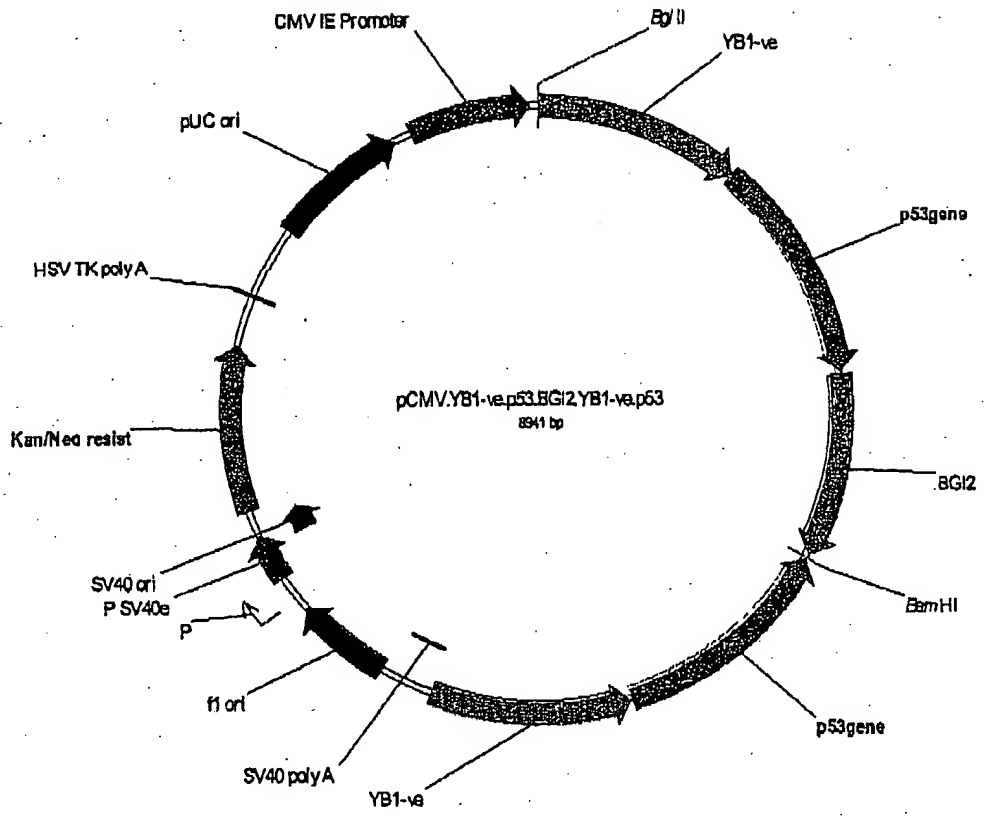
ANNEXURE MWG8



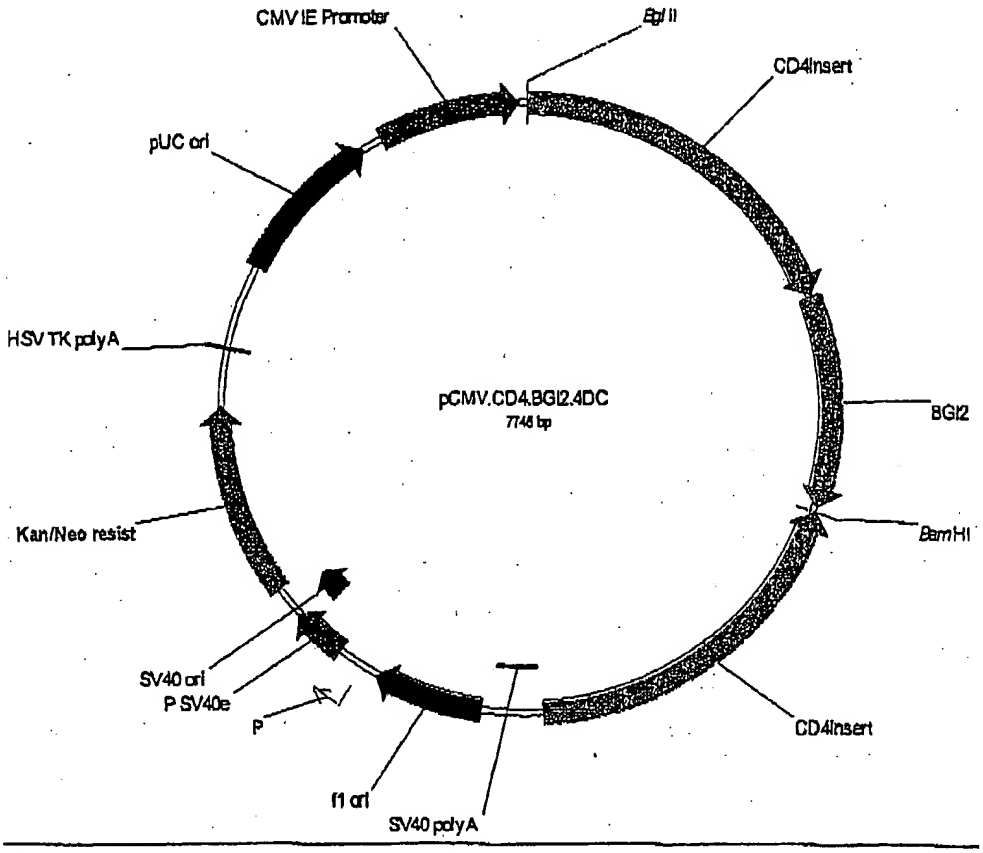
ANNEXURE MWG9



ANNEXURE MWG10



ANNEXURE MWG11



E.2

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Specific Double-Stranded RNA Interference in Undifferentiated Mouse Embryonic Stem Cells

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Specific mRNA degradation mediated by double-stranded RNA (dsRNA) interference (RNAi) is a powerful way of suppressing gene expression in plants, nematodes, and fungal, insect, and protozoan systems. However, only a few cases of RNAi have been reported in mammalian systems. Here, we investigated the feasibility of the RNAi strategy in several mammalian cells by using the enhanced green fluorescent protein gene as a target, either by in situ production of dsRNA from transient transfection of a plasmid harboring a 547-bp inverted repeat or by direct transfection of dsRNA made by in vitro transcription. Several mammalian cells including differentiated embryonic stem (ES) cells did not exhibit specific RNAi in transient transfection. This long dsRNA, however, was capable of inducing a sequence-specific RNAi for the episomal and chromosomal target gene in undifferentiated ES cells. dsRNA at 8.3 nM decreased the cognate gene expression up to 70%. However, RNAi activity was not permanent because it was more pronounced in early time points and diminished 5 days after transfection. Thus, undifferentiated ES cells may lack the interferon response, similar to mouse embryos and oocytes. Regardless of their apparent RNAi activity, however, cytoplasmic extracts from mammalian cells produced a small RNA of 21 to 22 nucleotides from the long dsRNA. Our results suggest that mammalian cells may possess RNAi activity but nonspecific activation of the interferon response by longer dsRNA may mask the specific RNAi. The findings offer an opportunity to use dsRNA for inhibition of gene expression in ES cells to study differentiation.

Frequently, inhibition of gene expression has been caused not only by antisense mRNA but also, in some cases, by expression of sense mRNA which has been used as a control. Moreover, this gene silencing by sense mRNA was shown to be sequence specific for the homologous gene. These initially confusing observations have now been attributed to gene silencing by the production of a minute amount of double-stranded RNA (dsRNA) generated by transcription of the sense mRNA by its promoter and the antisense mRNA by a cryptic promoter within the construct (reviewed in references 3, 6, 11, 26, 27, and 33). The term RNA interference (RNAi) was defined after the discovery that injection of dsRNA into the nematode *Caenorhabditis elegans* led to specific silencing of the gene homologous to the delivered RNA (12). RNAi was also observed in fruit flies, zebra fish, and other animals, including mice (7, 17, 30, 34, 35). The posttranscriptional gene silencing of *C. elegans* (18, 19, 28) is closely linked to the mechanism of cosuppression in plants and quelling in fungi (14, 21–23, 29).

Unlike other organisms, accumulation of very small amounts of dsRNA in mammalian cells results in the interferon response. This leads to an overall block of translation by inactivation of an elongation factor by protein kinase. In addition, dsRNA activates a latent 2′,5′-oligoadenylate synthase and increases synthesis of a 2′,5′-oligonucleotide, causing activation of RNase L and nonspecific mRNA degradation. These events result in the onset of apoptosis in mammalian cells (13, 16). The natural function of RNAi and cosuppression appears to be protection of the host genome against invasion by mobile genetic elements such as transposons and viruses, which produce dsRNA in host cells (14, 18, 20, 27). Such considerations have discouraged investigators from using RNAi in mammals. Recently, however, RNAi has been reported in several mammalian systems. Transfection of a plasmid carrying the full-length pro-α1(I) collagen gene into rodent fibroblasts decreased the endogenous pro-α1(I) collagen mRNA up to 90% (1). RNAi activity was also reported in CHO cells, although the amount of dsRNA required for interference was 2,500 times more than in *Drosophila* S2 cells (32). Sequence-specific RNAi has been demonstrated in the preimplantation mouse embryo and oocytes by direct injection of dsRNA (30, 35). When dsRNA corresponding to an active green fluorescent protein (GFP) gene was injected into mouse zygotes, dsRNAi was effective throughout the blastocyst stage and implantation until embryos reached 6.5 days of development, corresponding to a 40- to 50-fold increase in cell mass (35). With these findings, it becomes critical to determine whether RNAi can be applied in mammalian tissue culture for gene silencing.

The hallmark of RNAi is its specificity. The dsRNA triggers a specific degradation of homologous mRNA only within the region of identity with the dsRNA (37). The ability of a few molecules of dsRNA to eliminate a much larger pool of endogenous mRNA suggests a catalytic or amplification compo-

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nent to the RNAi mechanism. Results from studies of RNAi in plants suggested a mechanism, in which an RNA-primed RNA polymerase can spread gene silencing by dsRNA (28). Another model involves a catalytic RNA degradation generated by the dsRNA molecule and as yet unknown protein components. Recently, dsRNAs were shown to be processed to small 21- to 22-bp sizes in *Drosophila* embryo extracts (10, 36, 37), cultured S2 cells (2, 15), and *C. elegans* (24), making it likely that such RNAs serve as the specificity determinants in the RNAi reaction. These results suggest that dsRNA molecules are initially activated by a process that does not require interactions with their cognate mRNA target. Activation would appear to be a limiting step in RNAi, as the reaction is saturated at relatively low levels of dsRNA in vivo (12), potentiated by preincubation with dsRNA (31), and inhibited by excess unrelated dsRNAs (36).

Here, we investigated the feasibility of the RNAi strategy for gene silencing in several mammalian cell lines by using the enhanced GFP (EGFP) gene as a target. Our results show that undifferentiated mouse embryonic stem (ES) cells exhibit a sequence-specific RNAi at a dsRNA concentration similar to that needed in *Drosophila* S2 cells. We also compared the ability of different mammalian cell types to degrade dsRNA into small pieces of 21 to 22 bp, which is the initial step on RNAi activity.

MATERIALS AND METHODS

Plasmids. With pEGFP-C1 (Clontech, Palo Alto, Calif.) as the template, a 547-bp fragment encoding a portion of the EGFP gene beginning from the ATG start codon was amplified by PCR with the primers 5′-GCC GTC GAC GGT ACC TCT AGA ACG CGT GCC ATG GTG AGC AAG GGC GAG GAG-3′ and 5′-GCC GCG GCC GCG GCC CTA TTA GCC CTC GAG TAC ATG GTC GGC GAG CTG CAC GCT-3′. A set of restriction sites, *SalI*, *XbaI*, and *MluI* at the 5′ end and *EcoRI* and *NotI* at the 3′ end, were incorporated in each primer. After PCR amplification, the 547-bp fragment was digested with *NotI* and self-ligated. The dimer of the 547-bp fragment was isolated from the agarose gel, purified, ligated to the pGEMT Easy vector (Promega, Madison, Wis.), and used to transform *Escherichia coli* DH5α (Gibco-BRL, Rockville, Md.) competent cells. The resulting colonies were screened for inverted repeats by restriction enzyme analysis. The sequence of the plasmid harboring an inverted repeat (pGEMT-dsEGFP) was confirmed by DNA sequencing.

To generate control dsRNA, a 629-bp fragment encoding a portion of the *lacZ* gene (nucleotides 1331 to 1960 from the AUG start codon) was amplified by PCR and ligated to the pGEMT Easy vector. The colonies were screened for plasmids containing the insert in the sense and antisense orientations. The plasmid pSC6-T7-Neo, encoding the T7 RNA polymerase gene under the control of the cytomegalovirus (CMV) promoter was a generous gift from M. Billeter (25). The pActin-lacZ and pIZ/US9-GFP plasmids, encoding the *lacZ* and EGFP genes under the control of the *Drosophila* promoters for actin and OpIE2, respectively, were generous gifts from Gregory Hannon. The pCMV-lacZ plasmid was purchased from Clontech.

In vitro transcription of dsRNA. The pGEMT-dsEGFP construct with an inverted repeat containing a portion of the EGFP gene was linearized with *PstI* at a unique site located at the 3′ end of the inverted repeat. Using the RiboMax large-scale RNA production system-T7 (Promega, Madison, Wis.), the transcription reaction was performed at 37°C for 3 h, according to the manufacturer's specifications.

Radiolabeled dsRNA was generated by incorporation of [α-^{32}P]UTP during in vitro transcription. After performing the in vitro transcription reaction, RNase-free DNase (Promega, Madison, Wis.) was added to the reaction mixture at 1 U/μg of the template DNA and incubated for 15 min at 37°C. The transcript was further purified by extraction in phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitation. The pellet was washed with 70% ethanol, dried at room temperature, and resuspended in TE buffer (10 mM Tris [pH 7.5] and 1 mM EDTA). To determine the folded structure of the dsRNA, an aliquot of the RNA sample was digested using a mixture of RNase A and T_1 (Ambion, Austin, Tex.) at 37°C for 30 min and analyzed on 5% nondenaturing and denaturing polyacrylamide gels containing 40% formamide and 7 M urea. For dsRNA of the *lacZ* gene, plasmid containing either the sense or antisense *lacZ* fragment was linearized by restriction enzyme *SalI*, located downstream of the multiple cloning site. The sense and antisense RNAs were generated separately by in vitro transcription and annealed to generate a 740-bp dsRNA fragment.

Cell culture. *Drosophila* S2 cells (generous gift from G. J. Hannon) were maintained at 27°C in 90% Schneider's insect medium (Gibco-BRL, Rockville, Md.) and 10% heat-inactivated fetal bovine serum (FBS). Cells were split every 2 to 3 days to maintain exponential growth. BsrT7/5 (4), a derivative of DH1K-21 cells that express the T7 RNA polymerase (generous gift from M. Schnell), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and penicillin-streptomycin. CHO-K1 cells were maintained in F-12 medium with 10% heat-inactivated FBS. Mouse embryonic stem (ES) cells AB2.2 (Stratagene, La Jolla, Calif.) were maintained in DMEM supplemented with 1,250 U of leukemia inhibitory factor (LIF) (Chemicon, Temecula, Calif.) per ml, 15% FBS, 2 mM glutamine, 100 mM β-mercaptoethanol, and 1× nonessential amino acids. Mouse embryonic fibroblast STO cells (American Type Culture Collection, Rockville, Md.) were grown in DMEM with 10% FBS. The STO feeder cells were plated on dishes coated with 0.1% (wt/vol) gelatin, treated with mitomycin C (Sigma, St. Louis, Mo.) at a concentration of 10 μg/ml for 2.5 h at 37°C, and washed three times with phosphate-buffered saline (PBS). ES AB2.2 cells were plated onto mitomycin C-treated STO feeder cells and passaged every 2 days with a daily change of culture medium. For all experiments, ES cells were kept between 17 and 19 passages, counted from the time of isolation of ES cells from the inner cell mass of the blastocyst.

Transfection. The day before transfection, S2 cells were plated in a 12-well plate (10^6 cells per well). Various amounts of the *PstI*-linearized pGEMT-dsEGFP plasmid or dsRNA generated by in vitro transcription were combined with 1 μg of the target plasmid encoding EGFP (pEGFP-C1) and 1 μg of the plasmid encoding the T7 RNA polymerase (pSC6-T7-Neo). In all transfection experiments, a constant amount of total DNA, 5 μg, was maintained by addition of the unrelated pUC19 plasmid. DNA was transfected to S2 cells by the calcium phosphate method. The plasmid encoding β-galactosidase, pCMV-lacZ, was used as a control. CHO-K1 and STO feeder cells were plated in a 12-well plate (10^5 cells per well) the day before transfection. Various amounts of the *PstI*-linearized pGEMT-dsEGFP plasmid or in vitro-transcribed dsRNA was combined with 1 μg of the target plasmid encoding EGFP (pEGFP-C1) and 1 μg of the plasmid encoding the T7 RNA polymerase (pSC6-T7-Neo). The DNA mixture was transfected to cells by addition of 7.5 μg of Lipofectamine (Gibco-BRL, Rockville, Md.). The same transfection protocol was used for BsrT7/5 cells except the pSC6-T7-Neo plasmid was not added, as BsrT7/5 already expresses the T7 RNA polymerase (4).

ES cells were grown on feeder STO cells, trypsinized for 5 min, and pipetted extensively to prevent clumping of cells. After addition of 5 volumes of ES medium, cells were put back in the incubator for 45 min. The majority of the STO cells adhere to the plate during this incubation, and ES cells were harvested from the suspension. Various amounts of the *PstI*-linearized pGEMT-dsEGFP plasmid or dsRNA were combined with 1 μg of the target plasmid encoding EGFP (pEGFP-C1) and 1 μg of the plasmid encoding the T7 RNA polymerase (pSC6-T7-Neo). In all transfection experiments, a constant amount of total DNA, 5 μg, was maintained by addition of the unrelated pUC19 plasmid. A 150-μg M9 peptide (generous gift from Scott Diamond) was then added to the DNA solution in 100 μl of OptiMEM, and the DNA-M9 mixture was further incubated for 15 min at room temperature. Lipofectamine (7.5 μg) was diluted in 100 μl of OptiMEM and added to the DNA-M9 mixture for 45 min. The DNA-M9-Lipofectamine mixture was added to 3×10^5 ES cells in suspension and plated in a 12-well gelatin-coated plate containing 3×10^5 STO feeder cells pretreated with mitomycin C (Sigma, St. Louis, Mo.). The same procedure was used for transfection of ES cells without feeders except that ES cells were plated directly on gelatin plates without STO feeder cells, using medium without LIF.

Quantitation of EGFP and β-galactosidase activities. All adherent cells were harvested 72 h after transfection by washing with PBS and scraping cells into 100 μl of ice-cold lysate buffer (91.5 mM K_2HPO_4, 8.5 mM KH_2PO_4, and 1 mM dithiothreitol [DTT]). The harvested cells were then subjected to a dry-ice/ethanol freezing and thawing at 37°C for three cycles and centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was stored at −70°C until use. For ES cells with feeders, 72 h after transfection, cells were trypsinized for 5 min and pipetted extensively. After addition of 5 volumes of ES medium, cells were put back in the incubator for 45 min to allow the STO cells to attach to the plate. After this procedure, ES cells constituted more than 95% of the cells in suspension. ES cells were transferred to a tube, centrifuged, and processed the same way as other cells. The protein concentration of cell lysates was measured with the Pierce reagent (Pierce, Rockford, Ill.) in a 96-well plate. For each lysate, the same

amount of protein was used for the fluorescence and chemiluminescence measurements. Fluorescence was measured in relative light units (RLUs) using a 96-well black flat-bottomed plate (Corning Costar, Cambridge, Mass.) and an FL 600 microplate reader (Bio-Tek Instrument, Winooski, Vt.) with KC4 data reduction software on an external personal computer, which controls the reader function and data capture. Excitation was at 485 nm with a 20-nm band-pass filter, and emission was at 530 nm with a 25-nm band-pass filter. To account for the background, each fluorescence reading was subtracted from that of the untransfected cell lysate. The fluorescence reading of each lysate was normalized to that of the lysate prepared from cells transfected without dsRNA, either pGEMT-dsEGFP plasmid or in vitro-transcribed dsRNA.

β-Galactosidase activity was measured by histochemical staining and chemiluminescence. Cells were fixed with 1% glutaraldehyde and stained with X-Gal staining solution [0.1 M sodium phosphate (pH 8.0), 1.3 mM $MgCl_2$, 3 mM $K_4Fe(CN)_6$, 3 mM $K_3Fe(CN)_6$, and 0.4 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in *N,N*-dimethyl formamide] for 4 h. For chemiluminescence measurements, cell lysates were prepared using the Luminescent β-Galactosidase Genetic Reporter System II kit (Clontech, Palo Alto, Calif.). Lysates were analyzed in triplicate by chemiluminescence using the Tomtec LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). To account for the background, the chemiluminescence reading was subtracted from that of the untransfected cell lysate. The RLUs of each lysate were normalized to that of the lysate prepared from cells transfected without dsRNA, either pGEMT-dsEGFP plasmid or in vitro-transcribed dsRNA.

Fluorescence microscopy of S2 and ES cells. For fluorescence microscopy, S2 cells (2×10^6 S2 cells/well of a six-well plate) were plated and transfected with 2.5 μg of pIZ/US9-GFP and an increasing amount, 0, 1.5, or 3.0 μg, of the in vitro-transcribed dsRNA by the calcium phosphate method. ES cells (6×10^5 cells/well of a six-well plate) were mixed with 2.5 μg of pEGFP-C1 plasmid and increasing amounts, 0, 1, and 2 μg, of the in vitro-transcribed dsRNA and plated on the STO feeder cells as described above. Fluorescence micrographs were taken 72 h after transfection.

Analysis of RNA by Northern blotting in ES cells. ES cells were transfected by three plasmids as described above. Total RNA was purified by the RNAeasy Mini kit (Qiagen, Valencia, Calif.) and quantitated by UV absorbance at 260 nm. A total of 25 μg of RNA was loaded into each lane in a 0.8% formaldehyde denaturing agarose gel, and the Northern blotting was performed using the NorthernMax kit (Ambion, Austin, Tex.). The EGFP probe was a 0.7-kb fragment generated by *Nhe*I and *Bgl*II restriction enzyme digestions of plasmid pEGFP-C1, and the *lacZ* probe was a 2.5 kb fragment prepared by *Pvu*II digestion of the pCMV-lacZ plasmid. The probes were labeled by [α-^{32}P]dCTP using the Megaprime DNA labeling system (Amersham, Piscataway, N.J.). A cDNA probe corresponding to the mouse β-actin coding sequence was hybridized as a control.

Generation of ES cells with integrated EGFP gene, transfection with dsRNA, and FACS analysis. To produce ES cells with an integrated EGFP transgene, 2.5×10^6 AB2.2 cells were transfected with 6 μg of linearized pcDNA3-EGFP by M9 lipofection. G418 (275 μg/ml) selection was started 24 h after transfection. After 10 days of selection with G418, the surviving ES colonies were examined by fluorescence microscopy. Fluorescent colonies were picked and expanded according to established techniques. The number of integrated copies of pcDNA3-EGFP was determined by Southern blot analysis using the EGFP coding region as a probe. Several ES clones with a single copy of the EGFP gene were chosen for use in this study. ES cells were seeded at 10^5 cells/well of six-well plate in the presence and absence of the feeder layer and transfected with 3 μg of in vitro-transcribed dsRNA-EGFP or dsRNA-lacZ using 1.5 μg of Lipofectamine 2000 (Gibco-BRL, Rockville, Md.). The transfected cells were maintained with daily changes of medium and harvested at various time points to measure GFP fluorescence. Cells were trypsinized, centrifuged, suspended in chilled PBS, and subjected to fluorescence-activated cell sorting (FACS) analysis using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). Instrument settings were adjusted to separate live from dead cells, and fluorescence intensity data for 20,000 live cells were collected for each experimental time point. The relative levels of fluorescence for different samples were compared using the geometric means. Instrument settings were kept constant for all samples within each experiment. Data were analyzed using Cell Quest Software (Becton Dickinson).

Generation of small RNA fragments from dsRNA. Cytoplasmic extracts were isolated as described previously (8). Extracts were prepared from cells in the log phase of growth, and cytoplasmic proteins were extracted in a buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF; Sigma, Saint Louis, Mo.), and 0.5 mM DTT (Sigma, Saint Louis, Mo.). The final dialysis was performed for 12 h in an excess volume of dialysis buffer (20 mM HEPES [pH 7.9], 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT). The protein concentration was measured by the Bradford assay. Cytoplasmic extracts (10 to 50 μg) were incubated with 30 nmol of radiolabeled dsRNA for 1 h at 30°C for S2 cells or 37°C for mammalian cells. The standard reaction was carried out in a 20-μl reaction buffer containing 20 mM HEPES, 2 mM magnesium acetate, 2 mM DTT, 1 mM ATP, 40 mM creatine phosphate, and 100 μg of creatine phosphokinase and 1 U of RNasin (Ambion, Austin, Tex.) per ml. After the reaction, samples were treated with proteinase K (1 mg/ml)–0.5% sodium dodecyl sulfate (SDS) and purified by phenol-chloroform extraction. The size of the dsRNA was examined by a 12% denaturing acrylamide gel. After completion of electrophoresis, the gel was stained with ethidium bromide. The gel was then fixed in a 30% methanol 7% acetic acid solution, dried, and exposed to X-ray film at −80°C.

RESULTS

Generation of dsRNA for EGFP gene. To generate dsRNA in mammalian cells in situ, we cloned an inverted repeat of a portion of the EGFP gene, extending from the ATG codon to nucleotide 547, under the control of the T7 RNA polymerase (pGEMT-dsEGFP). Using this plasmid, we investigated the feasibility of the RNAi strategy for gene silencing in several mammalian cells by using the EGFP gene as a target. Two strategies were used: (i) in situ production of dsRNA by transient transfection of three plasmids, the target plasmid encoding the EGFP gene (pEGFP-C1), the plasmid harboring a 547-bp inverted repeat of the EGFP gene under control of the T7 promoter (pGEMT-dsEGFP), and the plasmid containing the T7 RNA polymerase cDNA under the control of the CMV promoter (pSC6-T7-Neo) (Fig. 1A), and (ii) direct transfection of in vitro-transcribed dsRNA (547 bp) and the target plasmid pEGFP-C1 (Fig. 1B).

The production of dsRNA was confirmed by in vitro transcription of the pGEMT-dsEGFP plasmid by the T7 RNA polymerase. Nondenaturing gel electrophoresis revealed a transcript of approximately 550 bp that did not change in size appreciably upon RNase A and RNase T_1 digestion, consistent with a double-stranded structure (Fig. 1C). When the RNA was analyzed in a 5% denaturing acrylamide gel (7 M urea–40% formamide), as expected, the size of the transcript was twice that in the nondenaturing gel (Fig. 1D). Upon RNase digestion, the fragment migrated predominantly to 550 nucleotides in denaturing conditions, indicating cleavage at the connecting loop of the folded dsRNA (Fig. 1D). These results confirm the generation of the 547-bp dsRNA by transcription of the pGEMT-dsEGFP plasmid. As a control, dsRNA for *lacZ* (740 bp) was generated by annealing the sense and antisense transcripts (Fig. 1E).

Sequence-specific gene silencing by production of dsRNA in situ in S2 cells. To investigate whether production of a dsRNA in situ by pGEMT-dsEGFP plasmid is sufficient to induce RNAi, we transfected S2 cells with three plasmids: pEGFP-C1, pSC6-T7-Neo, and increasing amounts of pGEMT-dsEGFP. When this plasmid is cotransfected with the gene encoding the T7 RNA polymerase under the control of the CMV promoter, it is expected to make dsRNA of 547 bp in mammalian cells. To test the specificity of RNAi, the plasmid encoding the β-galactosidase, pCMV-lacZ, was added instead of pEGFP-C1 in the control experiment, where all other reagents were kept the same.

Transfection of the pGEMT-dsEGFP plasmid showed a sequence-specific and dose-dependent inhibition of EGFP expression (Fig. 2A). In contrast, β-galactosidase expression was

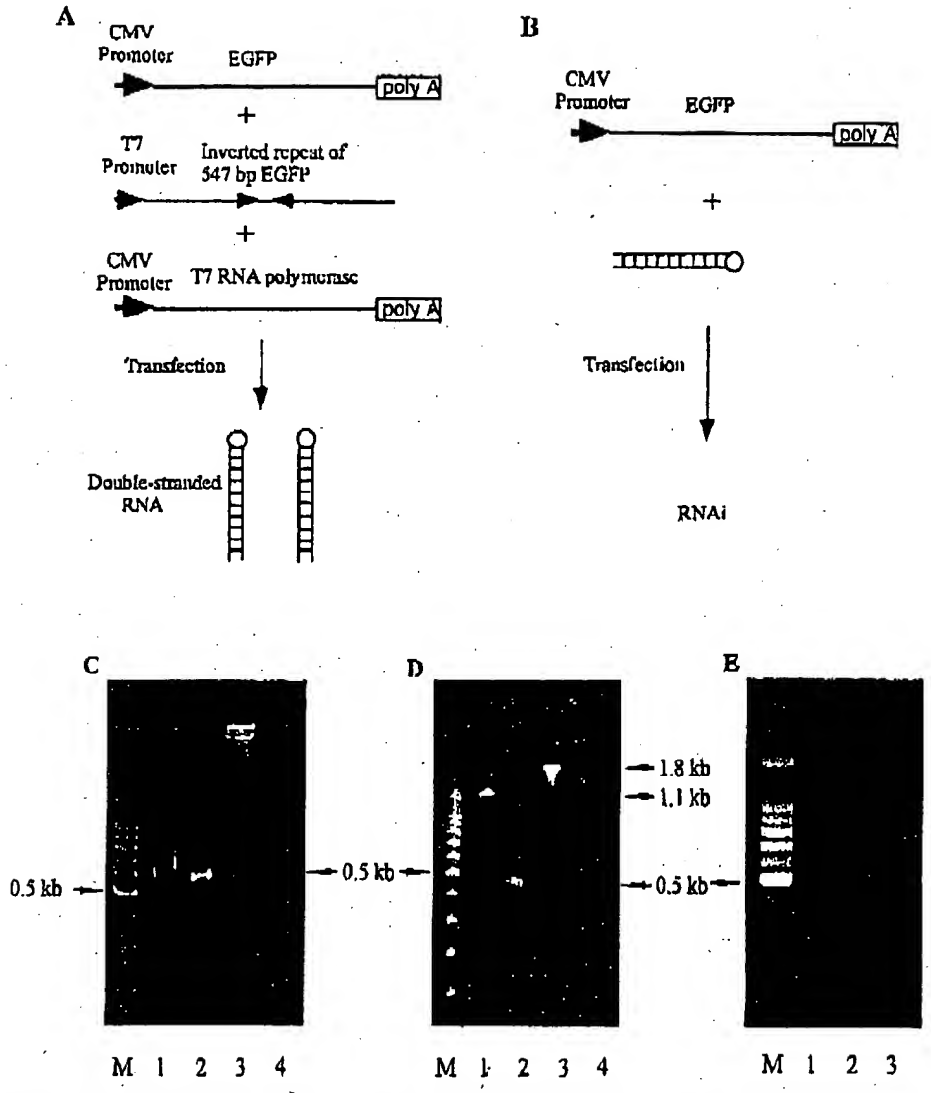

FIG. 1. Strategy for generation of dsRNA. (A) In situ production of dsRNA of EGFP. An inverted repeat of EGFP, starting from the ATG codon to nucleotide 547 in the coding region, was cloned into the pGEMT vector under control of the T7 promoter (pGEMT-dsEGFP). RNAi activity in mammalian cells was tested by transient transfection of three plasmids, the target plasmid encoding the EGFP gene, the linearized pGEMT-dsEGFP, and plasmid encoding the T7 RNA polymerase cDNA. (B) Direct transfection of dsRNA made by in vitro transcription. Mammalian cells were transfected with dsRNA and the target plasmid encoding the EGFP gene to compare the RNAi activities between in situ production of dsRNA and in vitro-transcribed dsRNA. The dsRNA-EGFP was made by in vitro transcription using the T7 RNA polymerase and the linearized pGEMT-dsEGFP plasmid. (C) Analysis of dsRNA-EGFP transcribed by the T7 RNA polymerase. The pGEMT-dsEGFP plasmid was linearized by *Pst*I, located at the 3′ end of the inverted repeat, to generate a runoff transcript by the T7 RNA polymerase. The transcribed RNA was digested with a mixture of RNases A and T_1. The RNA was analyzed in a 5% nondenaturing acrylamide gel. Lane M, 100-bp dsDNA ladder. Lanes 1 and 2 depict in vitro-transcribed RNA with and without RNase treatment, respectively. Lanes 3 and 4 show a control 1.8-kb RNA provided in the RiboMax kit, with and without RNase treatment, respectively. (D) The same RNA samples were electrophoresed on a 5% denaturing acrylamide gel containing 40% formamide and 7 M urea. (E) Analysis of the dsRNA-lacZ transcribed by the T7 RNA polymerase. The sense and antisense RNAs were transcribed from the plasmid linearized by *Sal*I and annealed to make dsRNA. Lanes 1 and 2 depict in vitro-transcribed antisense and sense RNAs, and lane 3 depicts annealed dsRNA in a 1% agarose gel.

not affected by addition of the pGEMT-dsEGFP plasmid, demonstrating the sequence-specific RNAi. The CMV promoter was not as strong as the *Drosophila* promoters actin or OpIE2 in S2 cells (data not shown). However, a sufficient amount of protein was generated to measure the EGFP and β-galactosidase activities. S2 cells were also used to compare the efficiency between the in situ production of dsRNA and the direct transfection of dsRNA made by in vitro transcription.

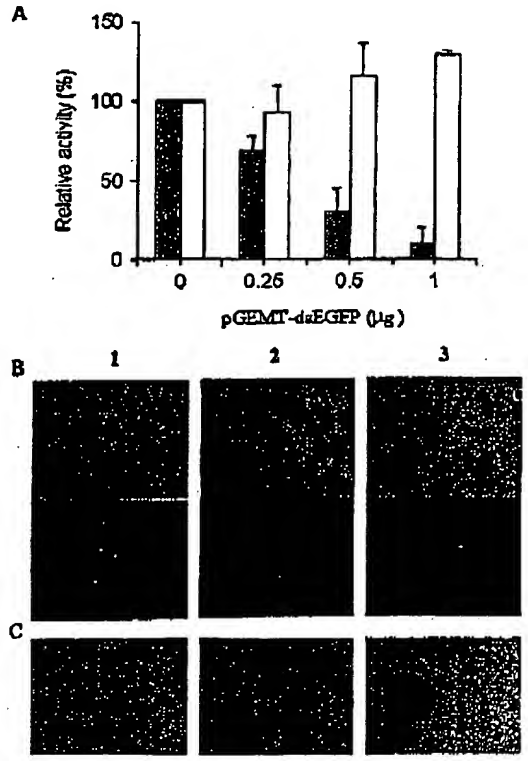

FIG. 2. dsRNA produced a sequence-specific and dose-dependent gene silencing in *Drosophila* S2 cells. (A) Inhibition of EGFP expression by in situ production of dsRNA. S2 cells were transfected with three plasmids, pEGFP-C1 (1 μg), pSC6-T7-Neo (1 μg), and an increasing amount of pGEMT-dsEGFP, ranging from 0.25 to 1 μg. Throughout all transfections, the total amount of DNA was held constant by addition of unrelated pUC19 plasmid. To test the sequence specificity of RNAi, 1 μg of the plasmid encoding *lacZ*, pCMV-lacZ, was used instead of pEGFP-C1. The RLUs of fluorescence or chemiluminescence were normalized to that of lysate containing no pGEMT-dsEGFP plasmid. The relative activities of cells transfected with pEGFP-C1 plasmid (solid bars) and pCMV-lacZ plasmid (open bars) are shown. Standard deviation indicates the variation among at least three separate transfection experiments performed in duplicate. (B) Sequence-specific and dose-dependent inhibition of EGFP by the in vitro-transcribed dsRNA. S2 cells were transfected with 2.5 μg of pIZ/US9-GFP plasmid and 0, 1.5, or 3.0 μg of the in vitro-transcribed dsRNA-EGFP (lanes 1, 2, and 3, respectively) using a calcium phosphate method. Photographs were taken 72 h later, depicted by a bright field (upper panel) and a fluorescence micrograph (lower panel). (C) β-Galactosidase expression is not inhibited by in-vitro transcribed dsRNA-EGFP. As a control, S2 cells were transfected with 2.5 μg of pActin-lacZ and 0, 1.5, or 3.0 μg of the in vitro-transcribed dsRNA-EGFP by a calcium phosphate method. Histochemical staining was carried out 72 h later.

Transfection of pIZ/US9-EGFP (the plasmid encoding EGFP under the control of the *Drosophila* promoter OpIE2) and an increasing amount of dsRNA, ranging from 0 to 3.0 μg, resulted in sequence-specific and dose-dependent inhibition of EGFP expression (Fig. 2B). Again, β-galactosidase expression was not affected by dsRNA (Fig. 2C). These results demonstrate that production of dsRNA in situ by the pGEMT-dsEGFP plasmid is sufficient to produce RNAi in S2 cells.

Several mammalian cells do not exhibit sequence-specific RNAi activity. We investigated the feasibility of the RNAi strategy for gene silencing in several mammalian cells by using the EGFP gene as a target and transient transfection of three plasmids, pEGFP-C1, pSC6-T7-Neo, and pGEMT-dsEGFP. For most experiments, 10^5 cells were plated on a 12-well plate and transfected with 1 μg of pEGFP-C1, 1 μg of pSC6-T7-Neo, and an increasing amount of pGEMT-dsEGFP, ranging from 0.25 to 2 μg. Because BsrT7/5 cells already express the T7 RNA polymerase (4), transfection was carried out under identical conditions except that the pSC6-T7-Neo plasmid was omitted. Two cell lines, BsrT7/5 cells and mouse fibroblasts (STO), showed a non-sequence-specific inhibition by dsRNA, indicated by reduction of both EGFP and β-galactosidase activities as increasing amounts of pGEMT-dsEGFP were added (Fig. 3A and 3B). The CHO-K1 cells did not exhibit any inhibition by dsRNA in cognate (EGFP) or noncognate β-galactosidase genes (Fig. 3C). In all experiments, we detected no apparent cytotoxicity, as measured by cell numbers and morphology (data not shown).

Undifferentiated ES cells exhibit sequence-specific RNAi activity. Recently, sequence-specific RNAi has been demonstrated in the preimplantation mouse embryo and mouse oocytes by direct injection of dsRNA (30, 35). However, dsRNA in transgenic blastocysts injected as zygotes produced gene silencing for only up to six rounds of cell division (35). These results suggest that undifferentiated cells may have RNAi activity that disappears as the cells differentiate. Here, we investigated whether undifferentiated ES cells respond to dsRNA for gene silencing.

Transfection of pGEMT-dsEGFP plasmid into undifferentiated ES cells maintained on the STO feeder layer showed a sequence-specific and dose-dependent inhibition of EGFP expression, while β-galactosidase expression was not affected (Fig. 4A). In contrast, differentiated ES cells maintained without STO feeder cells showed nonspecific inhibition (Fig. 4B). These cells progressively lost refractive boundaries and flattened to form a patch of giant trophoblastlike cells (data not shown), while undifferentiated ES cells remain as small cells packed tightly in nests (Fig. 5). Direct transfection of the in vitro-transcribed EGFP dsRNA, ranging from 0 to 1.0 μg, also resulted in a dose-dependent and sequence-specific inhibition of EGFP, shown by the fluorescence measurement of cell lysate (Fig. 5A) and by fluorescence microscopy of transfected cells (Fig. 5B). In contrast, β-galactosidase expression was not affected, as measured by either chemiluminescence (Fig. 5A) or histochemical staining (Fig. 5C).

Thus, only ES cells maintained in an undifferentiated state responded to dsRNA for gene silencing. The sequence-specific inhibition of dsRNA was also shown by a decrease in EGFP mRNA but not β-galactosidase mRNA, as measured by Northern blot analysis of ES cells transfected under identical conditions, in which the protein activity was measured (Fig. 6). These results confirm degradation of cognate EGFP mRNA but not β-galactosidase mRNA.

To investigate RNAi of an integrated gene in ES cells, we generated several ES clones with a single copy of the EGFP gene. We found that dsRNA-EGFP but not dsRNA-lacZ in-

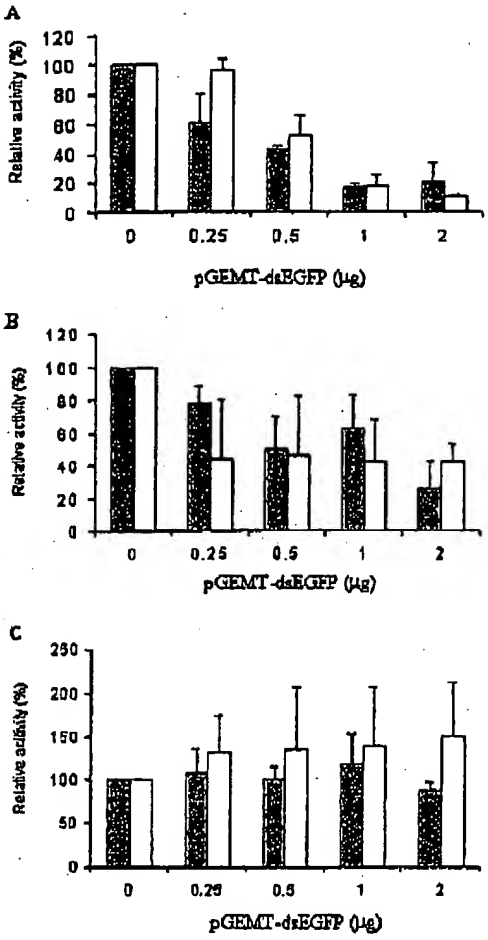

FIG. 3. Several mammalian cells do not show sequence-specific RNAi activity. Three mammalian cell lines, Hsr17/5 (A), STO (B), and CHO-K1 (C), were tested for RNAi activity by transient transfection of three plasmids, pEGFP-C1 (1 μg), pSC6-T7-Neo (1 μg), and increasing amounts of pGEMT-dsEGFP (0.25 to 2 μg). Throughout transfection, the total amount of DNA was held constant by addition of unrelated pUC19 plasmid. To test the sequence specificity of RNAi, 1 μg of the plasmid encoding the β-galactosidase, pCMV-lacZ, was used as a control. The RLUs of fluorescence or chemiluminescence were normalized to that of lysate containing no pGEMT-dsEGFP plasmid. The relative activities of cells transfected with pEGFP-C1 plasmid (solid bars) and pCMV-lacZ plasmid (open bars) are shown. Standard deviation indicates the variation among at least five separate transfections of duplicate samples.

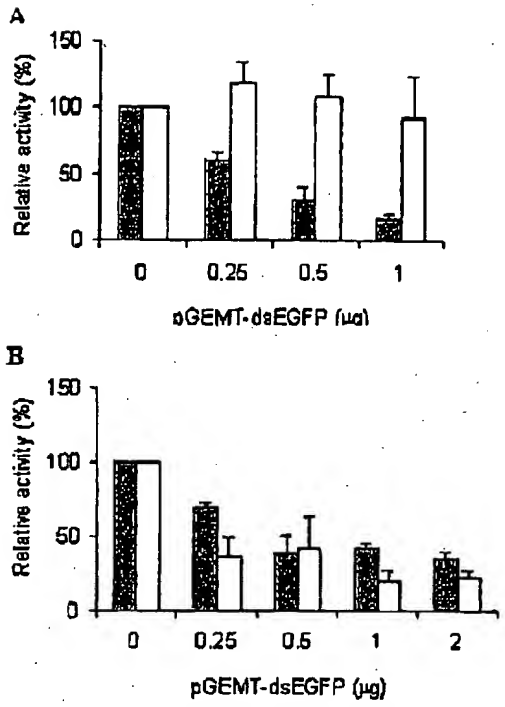

FIG. 4. Undifferentiated ES cells exhibit RNAi activity. (A) Sequence-specific and dose-dependent inhibition of EGFP by pGEMT-dsEGFP plasmid in ES cells grown on a feeder layer. ES cells were plated on STO feeder cells and transfected with three plasmids, pEGFP-C1 (1 μg), pSC6-T7-Neo (1 μg), and an increasing amount of pGEMT-dsEGFP, ranging from 0.25 to 1 μg. To test the sequence specificity of RNAi, 1 μg of the plasmid encoding β-galactosidase, pCMV-lacZ, was used as a control. The RLUs of fluorescence or chemiluminescence were normalized to that of lysate containing no pGEMT-dsEGFP plasmid. The relative activities of cells transfected with pEGFP-C1 plasmid (solid bars) and pCMV-lacZ plasmid (open bars) are shown. Standard deviation indicates the variation among at least five separate transfection experiments performed in duplicate. (B) Non-sequence-specific inhibition of EGFP by pGEMT-dsEGFP plasmid in differentiated ES cells cultured without the feeder layer. The same experiment was carried out in ES cells plated directly on a gelatin-coated plate with no feeder cells.

hibited EGFP gene expression among three different ES clones, as determined by fluorescence microscopy and FACS analysis. Transfection of dsRNA-EGFP but not dsRNA-lacZ resulted in a substantial decrease in fluorescence intensity of the ES cells (Fig. 7A). A representative FACS analysis of one of these clones is shown in Fig. 7B. The EGFP-positive cells were gated, and the relative fluorescence of each peak was measured using the geometric mean fluorescence. EGFP fluorescence decreased over 70% at 48 h after transfection of 8.3 nM dsRNA-EGFP but not by dsRNA-lacZ at the same concentration. Following transfection of dsRNA-EGFP, we observed a new population of cells with reduced fluorescence, indicated as M3 in the middle panel of Fig. 7B. The extent of inhibition was consistent among six independent transfections. Because only 20 to 30% of ES cells were transfected by Lipofectamine 2000 (unpublished observations), the large extent of inhibition by dsRNA suggests that dsRNA can be delivered efficiently to the cytoplasm and inhibits gene expression at a low concentration in mammalian cells (9).

We examined the kinetics of EGFP inhibition after transfection of dsRNA in undifferentiated ES cells. RNAi was more pronounced at early time points and diminished as undifferentiated ES cells replicated, presumably due to dilution of dsRNA per cell (Fig. 7C). Almost no inhibition was observed

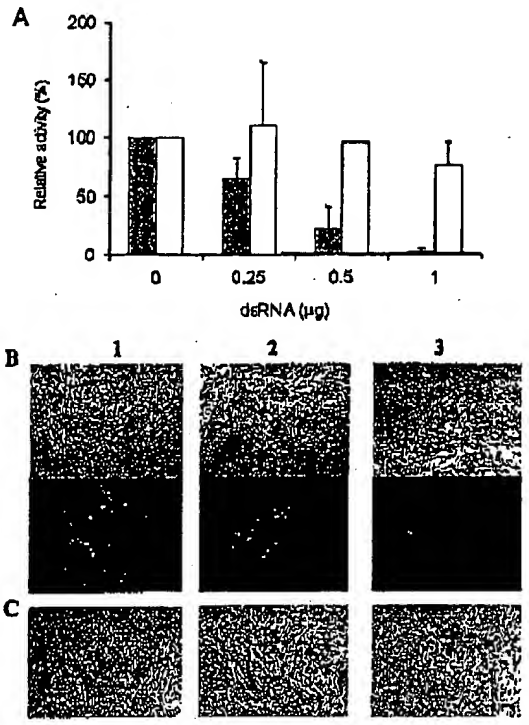

FIG. 5. Sequence-specific and dose-dependent inhibition of EGFP expression by in-vitro transcribed dsRNA in undifferentiated ES cells. (A) ES cells were plated on feeder cells and transfected with 1 μg of the pEGFP-C1 plasmid and increasing amounts, 0.25 to 1.0 μg, of the in vitro-transcribed dsRNA. To test the sequence specificity of RNAi, 1 μg of the plasmid encoding the β-galactosidase, pCMV-lacZ, was used as a control. The RLUs of fluorescence or chemiluminescence were normalized to that of lysate containing no dsRNA. The relative activities of cells transfected with pEGFP-C1 plasmid (solid bars) and pCMV-lacZ plasmid (open bars) are shown. Standard deviation indicates the variation among at least three separate transfection performed in duplicate. (B) Fluorescence microscopy of undifferentiated ES cells transfected with 2.5 μg of pEGFP-C1 plasmid and an increasing amount, 0, 1, and 2 μg (lanes, 1, 2, and 3, respectively), of the in vitro-transcribed dsRNA-EGFP. Photographs were taken 72 h later, using a bright field (upper panel) and fluorescence (lower panel). (C) β-Galactosidase expression is not inhibited by in vitro-transcribed dsRNA-EGFP. ES cells were transfected with 2.5 μg of pCMV-lacZ and 0, 1, or 2 μg of in vitro-transcribed dsRNA-EGFP. Histochemical staining was carried out 72 h later.

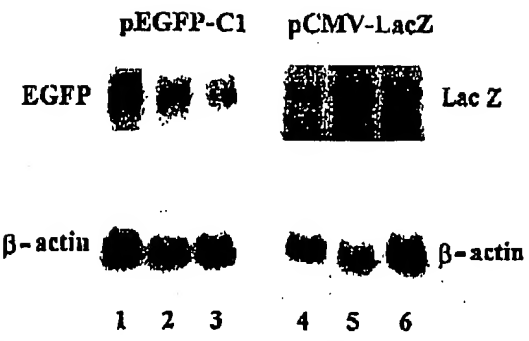

FIG. 6. Northern analysis of cognate (EGFP) and the noncognate (β-galactosidase) mRNAs. Undifferentiated ES cells were grown on the feeder layer and transfected by three plasmids, pEGFP-C1 (1 μg), pSC6-T7-Neo (1 μg), and an increasing amount of pGEMT-dsEGFP, 0, 1, or 2 μg (lanes 1, 2, and 3, respectively). As a control, ES cells were transfected with three plasmids, pCMV-lacZ (1 μg), pSC6-T7-Neo (1 μg), and an increasing amount of pGEMT-dsEGFP, 0, 1, or 2 μg (lanes 4, 5, and 6, respectively). Total RNA was isolated from transfected cells, and 25 μg of total RNA was loaded in each lane. The EGFP probe was a 0.7-kb fragment isolated from the pEGFP-C1 plasmid, and the *lacZ* probe was a 2.5-kb fragment from the pCMV-lacZ plasmid. The probes were labeled by [α-^{32}P]dCTP. A cDNA probe corresponding to the mouse β-actin coding sequence was hybridized as a control.

5 days after transfection. The stability of EGFP protein may account for the apparent lower inhibition at 24 h than 48 h. The dsRNA-lacZ did not show any inhibition of EGFP expression in all experiments, indicating specific gene silencing activity in undifferentiated ES cells. When the same ES cells were cultured without the feeder layer and LIF, ES cells did not completely differentiate. In this mixed population of ES cells, dsRNA-EGFP produced a reduction in fluorescence similar to that observed in undifferentiated ES cells, but dsRNA-lacZ did not.

The persistence of the RNAi effect in these experiments can be explained by the presence of a mixed population of differentiated and undifferentiated ES cells. However, it was difficult to measure the extent of gene silencing in fully differentiated ES-EGFP cells, since their intrinsic fluorescence decreased substantially upon differentiation. Further analysis of different clones is necessary to draw conclusions for RNAi for endogenous genes in differentiated ES cells. Taken together with the transient-transfection data described above, these results indicate that long dsRNA inhibited episomal and chromosomal target genes in undifferentiated ES cells in a sequence-specific manner.

dsRNA is processed to 21 to 22 nucleotides in mammalian cells. Small RNAs are associated with a dsRNA-dependent nuclease purified from cultured cells (15), making it likely that such RNAs serve as the specificity determinants in the RNAi reaction. Here, we investigated whether such dsRNA degradation activity may reflect the different RNAi activities among different mammalian cells. The dsRNA degradation activity was detected by the in vitro reaction in which a radiolabeled dsRNA was incubated with cytoplasmic extracts made from various cell types (Fig. 8). *Drosophila* S2 cells showed the highest activity, which was saturated between 10 and 50 μg of cytoplasmic protein (data not shown). Small RNA fragments were generated by all mammalian cell types tested: cells with a sequence-specific RNAi (undifferentiated ES cells), cells that showed no effect at all (CHO-K1), and cells that showed a nonspecific decrease in gene expression (differentiated ES, STO, and BsrT7/5 cells).

DISCUSSION

Posttranscriptional gene silencing by dsRNAi is a new tool for studying gene function in many organisms (3, 11, 26, 27, 33). However, only a few cases of RNAi have been reported in mammalian cells (1, 30, 32, 35). Here, we investigated the

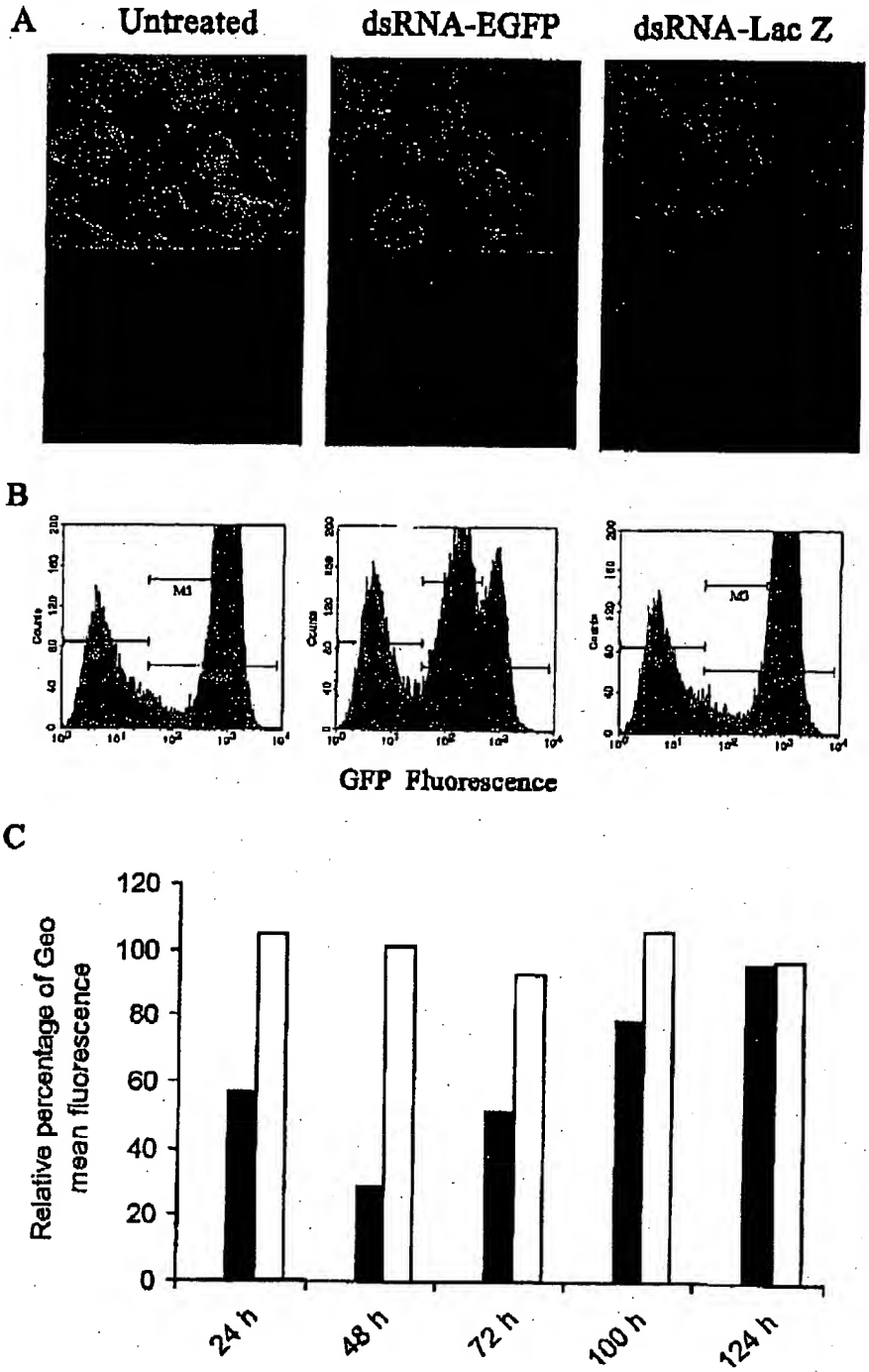

FIG. 7. Sequence-specific inhibition of EGFP expression by dsRNA in the stable ES-EGFP clone, in which the EGFP gene is integrated as a single copy. (A) Fluorescence microscopy of undifferentiated ES clone untreated or transfected with 3 μg of in vitro-transcribed dsRNA-EGFP or dsRNA-lacZ, respectively. Photographs were taken 72 h later, using a bright field (upper panel) and fluorescence (lower panel). (B) FACS analysis of the ES clone 48 h after transfection. M1 indicates the gating of GFP-negative cells, M2 the gating of GFP-positive cells, and M3 the gating of cells with reduced fluorescence due to RNAi. Untransfected clone (left panel; geometric mean fluorescence: M1 = 6.12, M2= 743.15), cells transfected with dsRNA-EGFP (middle panel; geometric mean fluorescence: M1 = 6.16, M2 = 270.93), cells transfected with dsRNA-lacZ

feasibility of the RNAi strategy for gene silencing in several mammalian cells by using the EGFP gene as a target, either by in situ production of dsRNA from a transient transfection of the plasmid harboring a 547-bp inverted repeat or by direct transfection of dsRNA made by in vitro transcription. In both cases, the dsRNA is generated as a hairpin structure that is resistant to RNase degradation. We reasoned that transient transfection may produce a large amount of stable dsRNA in the cytoplasm because it introduces a high copy number of the plasmid in the cytoplasm, which would then be transcribed by the T7 RNA polymerase. Using transient transfection, we show that undifferentiated ES cells have a sequence-specific RNAi activity that disappears as ES cells differentiate. This long dsRNA also inhibited its cognate gene expression in undifferentiated ES cells with a single integrated copy of the EGFP gene. Thus, both episomal and chromosomal target genes in undifferentiated ES cells were inhibited by the long dsRNA in a sequence-specific manner. Furthermore, the amount of dsRNA effective for RNAi activity in undifferentiated ES cells was similar to the amounts which caused gene silencing in *Drosophila* S2 cells and showed no apparent toxicity.

Several mammalian cell lines did not exhibit the sequence-specific gene silencing by dsRNA. Two cell lines, BsrT7/5 and mouse embryonic fibroblasts (STO), showed non-sequence-specific inhibition by dsRNA, while CHO-K1 did not exhibit any inhibition by dsRNA on either the cognate or noncognate gene, EGFP or *lacZ*. When transient cotransfection of plasmid DNA and dsRNA was carried out in several mammalian cells, 293 and NIH 3T3 cells showed no effect at all, while BHK cells showed a nonspecific decrease in gene expression (5). RNAi has been reported in CHO cells, although the amount of dsRNA required for interference was 2,500 times more than in *Drosophila* S2 cells (32). Because we tested RNAi under identical conditions in S2 and CHO cells, the amount of dsRNA needed to produce RNAi in the S2 cells used in our experiment may not be sufficient to produce RNAi in CHO cells. Recently, a longer dsRNA was shown to induce some sequence-specific silencing in addition to the nonspecific inhibition in mammalian cells (9). It is possible that the reporter system in this study is not sensitive enough to distinguish specific RNAi from the nonspecific inhibition. In all experiments, we detected no apparent cytotoxicity as measured by cell numbers and morphology.

Sequence-specific RNAi has been demonstrated in the preimplantation mouse embryo and mouse oocytes by direct injection of dsRNA (30, 35). The dsRNA in mammalian cells typically activates a protein kinase that phosphorylates and inactivates eIF2a (16). The ensuing inhibition of protein synthesis ultimately results in apoptosis. This sequence-independent response may reflect a form of primitive immune response, since the presence of dsRNA is a common feature of many viral life cycles. Mouse oocytes, however, clearly lack this response, as the oocytes injected with dsRNAs resume meiosis and mature to metaphase II (30). The preimplantation mouse embryo also lacks the response, as embryos injected with dsRNAs develop to the blastocyst stage (35). Specific RNAi activity present in undifferentiated ES cells suggests that undifferentiated ES cells may also lack an interferon response, similar to mouse embryos and oocytes (30, 35). However, RNAi activity was not permanent, since it was more pronounced at early time points and diminished as undifferentiated ES cells replicated, presumably due to dilution of dsRNA per cell.

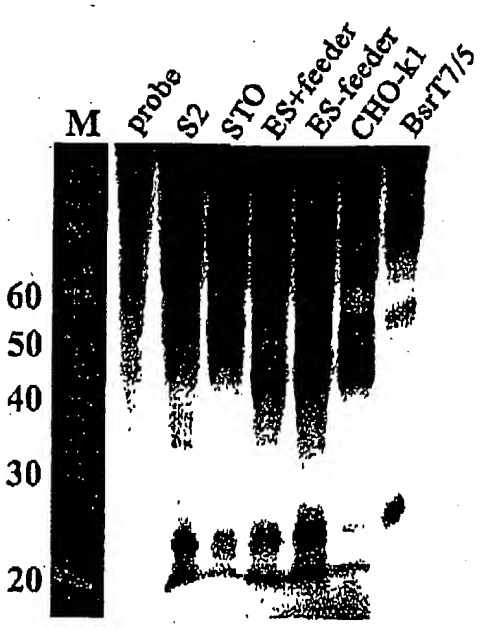

FIG. 8. Small RNA fragments generated by *Drosophila* S2 and mammalian cells. Cytoplasmic extracts (50 μg) from various mammalian cells were incubated with 30 nmol of radiolabeled dsRNA for 1 h at 30°C for S2 cells or 37°C for mammalian cells. After the reaction, samples were treated with proteinase K–0.5% SDS. The size of dsRNA was examined on a 12% denaturing acrylamide gel. Probe indicates the radiolabeled dsRNA made by in vitro transcription. Lane M, 10-bp markers.

Posttranscriptional gene silencing by dsRNA requires at least two steps, conversion of the dsRNA into an active species and subsequent targeting of the mRNA for inhibition by these sequence-specific active species. Recent biochemical studies (2, 10, 15, 31, 36, 37) have indicated that RNAi is accomplished by a multicomponent nuclease that targets cognate mRNA for degradation. The specificity of this complex was derived from the incorporation of a small guide sequence that is homologous to the mRNA substrate. These small 21- to 22-nucleotide RNAs, originally identified in plants with active RNAi (14), have also been observed in *Drosophila* embryos (10, 36, 37) and S2 cells (2, 15). We investigated whether such dsRNA degra-

(right panel; geometric mean fluorescence: M1 = 6.43, M2= 772.95). (C) Kinetics of RNAi in undifferentiated ES-EGFP clone. The relative geometric mean fluorescence of cells transfected with dsRNA-EGFP (solid bars) or dsRNA-*lacZ* (open bars) was normalized to the geometric mean fluorescence of untransfected cells. ES cells were split at 72 h after the initial transfection of dsRNA and plated at 2×10^5 cells/well for the later time measurements at 100 and 124 h.

dation activity may reflect the different RNAi activities among different mammalian cells.

Although *Drosophila* S2 cells showed the most prominent product of 21 to 22 bp, all mammalian cells tested produced distinct RNA products of the same size. Thus, mammalian cells have the ability to generate 21- to 22-nucleotide fragments from long dsRNA regardless of their apparent RNAi activity. While our manuscript was being reviewed, Tuschl's group reported that 21-nucleotide short interfering RNA (siRNA) was capable of gene silencing in several mammalian cells in which longer dsRNA failed to produce RNAi (9). The apparent lack of RNAi by longer dsRNA in mammalian cells was attributed to nonspecific activation of the interferon response by dsRNA longer than 30 bp, masking the specific RNAi. Therefore, our findings that mammalian cells can generate siRNA regardless of their apparent RNAi activity provide an insight in gene silencing of mammalian cells by siRNA. It would be interesting to compare the extent of gene silencing by siRNA and longer dsRNA in cells that do not show nonspecific inhibition.

ES cells and other stem cells are valuable tools for the study of cell and tissue differentiation and for the creation of animal models of disease. These findings offer an opportunity to use dsRNAi for inhibition of gene expression in ES cells to study differentiation. Stem cells also have the potential for therapeutic use, including the development of replacement tissues if regulation of their differentiation can be understood. The results presented here indicate that RNAi can be used to inhibit gene expression in mouse ES cells and thus may be a useful approach for investigations of stem cell biology in general.

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E3

letter

Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes

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Double-stranded RNA interference (RNAi) is an effective method for disrupting expression of specific genes in *Caenorhabditis elegans* and other organisms[1–5]. Applications of this reverse-genetics tool, however, are somewhat restricted in nematodes because introduced dsRNA is not stably inherited[5]. Another difficulty is that RNAi disruption of late-acting genes has been generally less consistent than that of embryonically expressed genes, perhaps because the concentration of dsRNA becomes lower as cellular division proceeds or as developmental time advances[1]. In particular, some neuronally expressed genes appear refractory to dsRNA-mediated interference. We sought to extend the applicability of RNAi by *in vivo* expression of heritable inverted-repeat (IR) genes. We assayed the efficacy of *in vivo*-driven RNAi in three situations for which heritable, inducible RNAi would be advantageous: (i) production of large numbers of animals deficient for gene activities required for viability or reproduction; (ii) generation of large populations of phenocopy mutants for biochemical analysis; and (iii) effective gene inactivation in the nervous system. We report that heritable IR genes confer potent and specific gene inactivation for each of these applications. We suggest that a similar strategy might be used to test for dsRNA interference effects in higher organisms in which it is feasible to construct transgenic animals, but impossible to directly or transiently introduce high concentrations of dsRNA.

To test the feasibility of specific gene disruption via *in vivo* expression of dsRNA, we constructed transgenic nematodes that synthesized hairpin dsRNA (ref. 3) from IR genes under the control of the heat-shock–inducible promoter *hsp16-2* (Fig. 1; refs 6–8). We first compared effects of conventional RNAi via injection of dsRNA, expression of sense and antisense genes, and *in vivo* production of dsRNA using *C. elegans* predicted gene C37A2.5, which is essential for progression past the L2 larval stage (N.T., S.L.W. and M.D., unpublished data). Conventional RNAi through injection of C37A2.5 dsRNA (ref. 1) produced a high yield of L2-stage–arrested F1 progeny (Table 1). Expression of the antisense strand, which can be effective for specific gene inactivation[9], resulted in a modest percentage of phenocopy progeny, whereas expression of the sense stand was ineffective. To test *in vivo* RNAi, we heat-shocked young adults of transgenic lines harbouring extrachromosomal *hsp16-2*$_p$C37A2.5(IR). *In vivo* promoter-driven RNAi reproduced the ΔC37A2.5 null phenotype, with efficiencies approaching that of direct injection of dsRNA (Table 1). Likewise, promoter-driven RNAi disrupted the Mi-2 chromatin remodelling homologue F2612.7 (ref. 10) to phenocopy the sterile phenotype of a deletion of this gene (Table 1). We concluded that *in vivo*-driven RNAi is effective, and that this technique should enable generation of large populations of phenocopy mutants, even when development or reproduction is blocked.

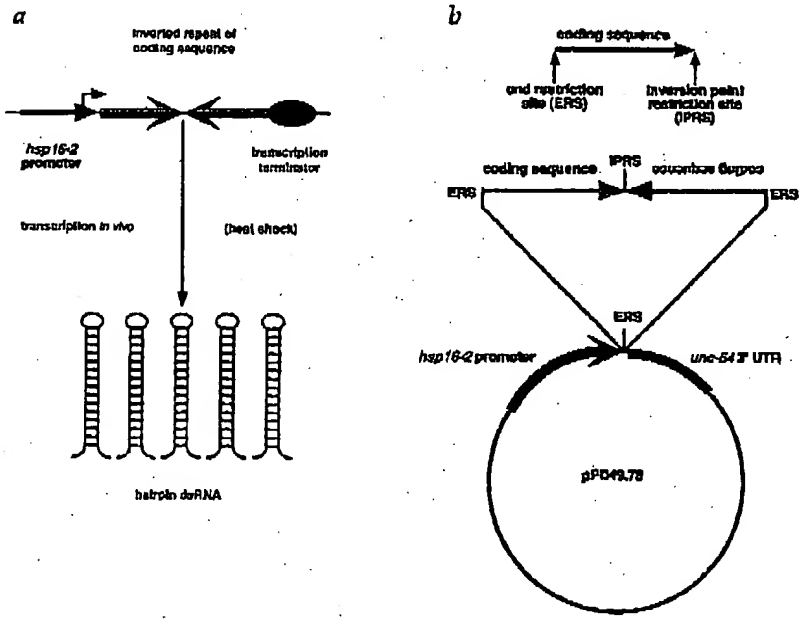

Fig. 1 Strategy for generation of heritable and inducible RNAi. *a*, A strategy for *in vivo* RNAi. A strong heat shock–inducible promoter was fused to a direct IR gene. Upon heat shock of transgenic animals harbouring this gene, transcripts were generated which are predicted to fold back in a uni-molecular reaction to generate double-stranded RNA in all cells that express the heat-shock gene. The size of the single-stranded loop that occurs after foldback is not known. *b*, Construction of inducible IR genes. Exon-rich genomic DNA (or cDNA) was amplified using two primers that introduced unique restriction sites at the fragment ends. One restriction site was used to generate the IR and was ultimately situated at the inversion point (IP). The other restriction site (designated as end) was used to join the IR to the vector. Amplified fragments were digested with the enzyme situated at the IP restriction site (IPRS) and ligated together. Digestion at the end restriction site (ERS) enabled the fragment to be cloned into a similarly digested, CIAP-treated *C. elegans* expression vector. We used vector pPD49.78 (ref. 22), which includes the *hsp16-2* promoter and the 3′ UTR of muscle myosin *unc-54*.

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Table 1 • *In vivo* dsRNA interference

Gene disruption approach	Trial/line 1	Trial/line 2	Trial/line 3	Trial/line4
dsRNA C37A2.5 injected	94±8	89±4	97±5	89±7
pPD49.78 (*hsp16-2*$_p$ alone) + heat shock	0	0	0	0
hsp16-2$_p$C37A2.5 sense + heat shock	0	0	0	0
hsp16-2$_p$C37A2.5 antisense + heat shock	9±4	9±4	11±6	–
hsp16-2$_p$C37A2.5(IR) – heat shock	0	0	0	0
hsp16-2$_p$C37A2.5(IR) + heat shock	67±3	79±6	84±5	56±7
hsp16-2$_p$F26F12.7(IR) – heat shock	1±0.9	2±1	1±0.9	3±1.3
hsp16-2$_p$F26F12.7(IR) + heat shock	58±4	59±5	75±8	82±6
ds *mec-4* RNA injected	12±7	19±5	15±6	–
hsp16-2$_p$*mec-4*(IR) – heat shock	0	0	0	–
hsp16-2$_p$*mec-4*(IR) + heat shock	58±4	60±7	61±8	–
ds *unc-8* RNA injected	0	0.8±.01	0	0
hsp16-2$_p$*unc-8*(IR) – heat shock	0	0	0	0
hsp16-2$_p$*unc-8*(IR) + heat shock	17±3	11±5	14±2	13±3

Results for four injection trials using conventional RNAi or heat-shock–induced *in vivo* RNAi in four transgenic lines (unless otherwise noted) are indicated. Numbers indicate the percentage of F1 progeny arrested at the L2 stage ±s.d.

C. elegans translation elongation factor 2 kinase eEF-2 (encoded by *efk-1*; ref. 11) phosphorylates eEF-2, an activity abolished by a Tc1 insertion into the active site (Fig. 2*a*, and A.R., C. Mendola, L. Zhang and J. Culotti, unpublished data). We found that kinase activity in the offspring of heat-shocked *hsp16-2*$_p$*efk-1(IR)* transgenic parents was reduced in four of six lines assayed. We were not able to perform an analogous assay on a population of phenocopy mutants induced by conventional RNAi, as several hundred animals were required. We concluded that inducible IR genes are effective in generating populations amenable to biochemical analysis.

Injected dsRNA is not uniformly effective in disrupting gene expression in the nervous system. For example, we found that only 6 of 210 progeny from three lines harbouring integrated *unc-119*$_p$*GFP* (expressed in all neurons) injected with double-stranded *GFP* RNA showed reduced fluorescence (Fig. 2*b*). We therefore examined the effects of endogenously expressed dsRNA species on gene inactivation in the differentiated nervous system. We constructed a plasmid that directed *in vivo* expression of double-stranded *GFP* RNA upon heat shock and tested for extinction of fluorescent signals generated by cell-specific *GFP* reporter fusions (Fig. 2*b*). We co-introduced the *hsp16-2*$_p$*GFP(IR)* construct and *unc-119*$_p$*GFP* (pIM175 (ref. 12); expressed at high levels throughout the nervous system[13]), selected lines exhibiting strong GFP fluorescence, heat shocked in the L4 stage and examined fluorescence in their progeny. Approximately 79% of roller progeny from 3 (of 5) lines harbouring *unc-119*$_p$*GFP* and *hsp16-2*$_p$*GFP(IR)* exhibited 'knock-down' effects, with fewer than 10 cells detectable in most (Fig. 2*b*). We did not detect any consistent pattern of cells that appeared refractory to fluorescence inactivation, suggesting that all cells in the nervous system are susceptible to the effects of *in vivo* RNAi.

We next tested effects of heat-shock induction of *hsp16-2*$_p$*GFP(IR)* on expression of an integrated *mec-4*$_p$*GFP* gene, which is specifically expressed in the six touch-receptor neurons[14]. On average, 85% of roller progeny of heat-shocked parents harbouring the extragenic *hsp-16*$_p$*GFP(IR)* transgene had GFP signals that were either eliminated or attenuated (2/4 lines; Fig. 2*b*). We observed similar effects in only 11 of 270 progeny of a line harbouring an integrated *mec-4*$_p$*GFP* reporter injected with dsGFP RNA.

We also tested for dsRNA-mediated inactivation of *C. elegans* neuronal genes. Conventional RNAi mediated by introduced *mec-4* dsRNA induced touch-insensitivity in 46 of 300 (15%) offspring of injected wild-type parents. On average, 60% progeny of heat-shocked lines harbouring *hsp16-2*$_p$*mec-4(IR)* were insensitive to touch (Table 1). Additionally we tested the effectiveness of *in vivo*-directed RNAi in the inactivation of *unc-8*, a neuronally expressed gene that we have found to be resistant to the effects of conventional RNAi. The *unc-8* dominant gain-of-function allele *n491* induces uncoordinated locomotion characterized by the inability to back up; the loss-of-function phenotype is nearly wild type[15]. Injection of *unc-8* dsRNA did not disrupt the gain-of-function phenotype (2 phenocopy mutants generated among 1,300 progeny of injected parents). By contrast, 13% of the progeny of heat shocked unc-8(n491) parents harbouring *hsp16-2*$_p$*unc-8(IR)* were effectively targeted (Table 1). Our results indicate that sequences expressed in terminally differentiated neurons can be targeted by *in vivo*-induced RNAi, and in some instances effects are more potent than those observed after injection of dsRNA.

For all nine cases, heat shock of control lines carrying the expression vector alone or low-temperature growth of lines carrying the *hsp16-2*$_p$*(IR)* genes did not produce any abnormal phenotypes (we assayed for the anticipated knockout phenotype, morphological and locomotion defects, and fertility and developmental abnormalities; >100 animals examined per line). Thus, effects of *in vivo* RNAi appear to be highly specific, consistent with reported tight regulation of the *hsp16-2* promoter[8] and the selective precision of RNAi (ref. 1). Moreover, *in vivo* RNAi was effective in many tissue types, including neurons (Fig. 2*b*). (C37A2.5 and *efk-1* are expressed early in development and later in a broad range of cells including body wall and pharyngeal muscles, neurons, hypodermis and intestine (N.T., A.R. and M.D., unpublished data); M12 homologue F26I2.7 is expressed in hypodermis; M12 homologue T14G8.1 is expressed in hypodermis and pharynx (S.L.W., N.T. and M.D. unpublished data); and *myo-2* is expressed in pharyngeal tissue[16].)

Our analysis establishes that endogenous IR genes can be expressed to generate dsRNA species with biological effects similar to, and in some cases more effective than, those of directly injected dsRNA. There are several advantages of expressing heri-

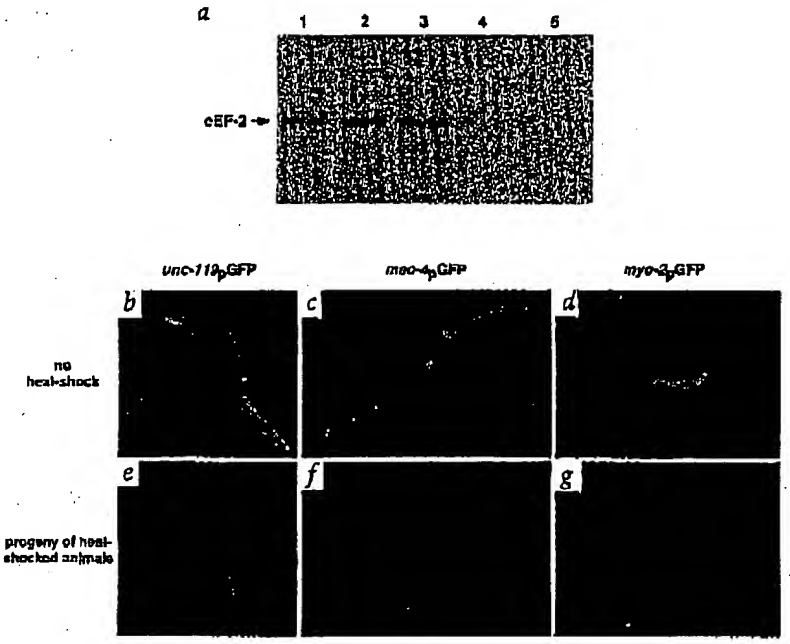

Fig. 2 Double-stranded RNA synthesized *in vivo* RNAi disrupts *C. elegans* gene expression. *a*, Enzymatic assay for elongation factor-2 kinase activity (EFK-1). EFK-1 activity was assayed as described[11] in reactions in which 0.5 µg rabbit reticulocyte eEF-2 was added to worm protein extracts. The arrow indicates the eEF-2 protein position. Lane 1, wild type; lane 2, line harbouring extrachromosomal *hsp16-2*$_p$*Cefk-1(IR)*, non-heat shocked; lane 3, a transgenic line harbouring extrachromosomal parental vector pPD49.78, heat shocked; lane 4, line harbouring extrachromosomal *hsp16-2*$_p$*Cefk-1(IR)*, heat shocked; lane 5, Tc1 active site insertion *Cefk-1* mutant. *b*–*g*, *In vivo* RNAi disrupts GFP expression in neurons and pharyngeal muscle. Progeny of transgenic lines harbouring extrachromosomal *unc-119*$_p$*GFP* (*b*,*e*; *unc-119* is expressed in all neurons[13]), integrated *mec-4*$_p$*GFP* (*c*,*f*; *mec-4* is expressed in six touch sensory neurons[14]) or *myo-2*$_p$*GFP* (*d*,*g*; *myo-2* is expressed in pharyngeal muscle[15]) and *hsp16-2*$_p$*GFP(IR)* were compared at 20 °C or consequent to parental heat shock at the L4 stage (35 °C, 4 h). Progeny of similarly heat-shocked *unc119*$_p$*GFP*, *mec-4*$_p$*GFP* or *myo-2*$_p$*GFP* lines exhibited no apparent reduction in intensity of neuronal fluorescence (data not shown). In parallel conventional RNAi experiments, 6 of 210 progeny of an *unc-119*$_p$*GFP* parent, 11 of 270 progeny of a *mec-4*$_p$*GFP* parent, and 57 of 240 progeny of a *myo-2*$_p$*GFP* parent exhibited detectable reduction in GFP signal.

table IR genes: (i) stable lines harbouring the potential for gene inactivation can be easily maintained; (ii) assays requiring large numbers of mutant phenocopies are feasible; and (iii) inhibition can be inducible, and thus may be used for stage-specific gene inactivation. In some cases, the endogenous high level of dsRNA product presumed produced upon heat shock appears to make for more potent inhibition than germline-injected dsRNA. Although we have focused our initial studies on the use of the inducible *hsp16-2* promoter, our findings suggest that it may be possible to inactivate specific genes for the duration of their expression period by integrating a transgene in which the promoter of the gene of interest drives transcription of an IR segment of the same gene. In addition, because dsRNA can inactivate genes in flies, plants, tryponosomes and planaria[17–21], and gene-based delivery can be effective in trypanosomes and plants [18,21], *in vivo*-directed RNAi may be effective in other organisms. A similar strategy for *in vivo*-driven RNAi might be successfully applied to inactivate specific genes in organisms that can be genetically engineered, but are not readily amenable to direct injection of dsRNA, such as the mouse.

Methods

Nematode strains. We reared and maintained *C. elegans* strains as described[24]. We constructed transgenic lines by injecting plasmid DNA (100 ng/µl) using standard protocols[23]. In all experiments we used plasmid pRF4 (*ref. 24*), which harbours a dominant *rol-6* allele that causes a readily distinguished roller phenotype in transgenic animals, as a co-transformation marker.

Construction of IR genes. We amplified by PCR exon-rich genomic DNA (or cDNA) using two primers that introduced unique restriction sites at the fragment ends. We digested the amplified fragment with one of the enzymes and ligated to generate an IR. We then digested with the other enzyme, the restriction site for which was positioned at the IR fragment ends, and ligated into ClAP-treated vector pPD49.78 (*ref. 23*), which includes the *hsp16-2* promoter and the 3′ UTR of muscle myosin *unc-54*. The cDNA and genomic DNA amplified for RNAi ranged from 0.58 to 1.45 kb. Alternative cloning strategies included digestion at two naturally occurring restriction sites to excise the gene fragment of interest with subsequent two-step ligation as above, or direct tri-molecular ligation of the doubly digested fragment into ClAP-treated vector previously linearized with one of the endonucleases at the fragment end. We found the efficiency of cloning inverted repeats to be low but acceptable in the *Escherichia coli* DH5α strain (in general, a few per hundred candidates screened) and relatively high in the *E. coli* SURE strain (Stratagene), a bacterial host tolerant of IRs (about 1/20 candidate constructs correct). The *hsp16-2*$_p$*unc-8(IR)* construct, however, was difficult to generate (1,000 candidates screened, 0.58 kb of cDNA sequence in the repeat) for reasons that are not clear. Slower-growing bacterial transformant colonies appear to have an enhanced chance of harbouring the IR gene. The yield of plasmid DNA from IR genes harboured in the *E. coli* DH5α strain was low (~3–5 µg per 50 ml culture); when the SURE strain was used as host, yields were improved (80–100 µg per 50 ml culture). Clone structures were verified using multiple restriction digests according to standard protocols.

RNA interference assays. For standard RNAi, we prepared dsRNA from cDNAs or coding sequence-rich genomic DNAs (0.58–1.2 kb) injected into N2 adults as described[1]. We scored progeny born to injected adults (10 adults per group) 12 h or more after injection (older progeny exhibit a lower phenocopy rate). For genetically directed RNAi mediated by expression of IR genes, we selected 50 transgenic roller L4s from lines harbouring various *hsp-16*$_p$*(IR)* constructs plus co-transformation plasmid pRF4 (array transmission frequency >60%; ref. 24) and reared continuously at 20 °C (non-heat shock), or heat shocked for 4 h at 35 °C, before returning to 20 °C. We scored progeny of these animals for phenotypes of interest at embryonic or larval stages as appropriate. In all experiments, at least 100 animals were scored per experimental trial. Co-expression of sense and antisense genes, which can be effective[25], was not tested. C37A2.5 is required for developmental progression past the L2 stage. Deletion of chromatin remodelling gene homologue F26F12.7 causes sterility (S.E.W. and M.D., unpublished data). Treated progeny of transgenic lines harbouring *hsp16-2*$_p$F26F12.7(IR) were scored for the percentage that failed to develop into fertile adults. A similar strategy for *in vivo* disruption of a second MI-2 homologue, T14G8.1, yielded 59% and 72% sterile in progeny of two lines scored after heat shock (data not shown). *mec-4* is expressed in six mechanosensory neurons and is required for touch sensi-

tivity. Double-stranded *mec-4* RNA or plasmid *hsp16-2$_p$mec-4(IR)* was introduced into wild-type animals and progeny were scored for touch insensitivity. *unc-8(n491)* is a dominant gain-of-function mutation that causes coiling and backward paralysis; locomotion in a loss-of-function mutant is nearly normal[15]. Double-stranded *unc-8* RNA or plasmid *hsp16-2$_p$unc-8(IR)* was introduced into the *n491* background and progeny were assayed for backing proficiency. Note that to regain backing ability, gene expression must be knocked down in most *unc-8*–expressing cells, ~60 neurons. On average at least half of lines for a given gene assayed conferred potent interference on heat activation.

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